# Morphological classification of soft-shell clam, *Mya arenaria*, hemocytes: phenotypic description of hemic neoplasia

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## ABSTRACT

In Atlantic Canada, hemic neoplasia (HN) has a negative impact on aquaculture and fisheries of the soft-shell clam, *Mya arenaria*. HN is a cellular disorder that morphologically and functionally alters the hemocytes. These abnormal hemocytes are found proliferating in the hemolymph and infiltrating tissues. Cells described as neoplastic are larger with a high nucleus to cytoplasm ratio compared to normal hemocytes. Although the morphology and behavior of neoplastic cells has been described, information regarding the characteristics of normal hemocytes in the soft-shell clam is lacking. The present study used cytology and transmission electron microscopy to characterize 5 types of hemocytes in clams. Hemolymph samples of normal clams had a mean differential hemocyte count of 62.6% agranular and 34.7% granular cells. Evaluation of the normal hemocytes provided descriptive information that was then useful for recognizing neoplastic cells. Two types of neoplastic cells and intermediate forms were observed at various levels of disease intensity. This study proposes criteria for recognizing neoplastic cells.

Flow cytometry is a technique to differentiate neoplastic from non - neoplastic cells in a non-lethal, labor efficient and reliable manner. Using the flow cytometer case definition for an individual clam positive for HN, clams from six populations in Atlantic Canada were statistically compared. Cytology was used for confirmatory purposes. Developing a case definition for a HN positive population was more challenging. Disease prevalence as well as intensity was considered. A sample population from Saguenay Bay, QC was considered negative because all clams were classified as negative on an individual basis. A sample population from North River, PEI had 35% prevalence and was the only population with high disease intensity; it was considered population positive for HN. The four other populations also had high prevalence but low intensity and were not able to be classified as disease positive or negative. The status of these populations should be checked on a seasonal basis to see if prevalence and intensity change.

# DEDICATION

To Paul,

*la raíz de mi árbol*

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### Chapter One



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### Chapter Three

## LIST OF ABBREVIATIONS

| | |
|---|---|
| ATP | Adenosine 5'-triphosphate |
| AMP | Antimicrobial peptides |
| ANOVA | Analysis of Variance |
| ° C | degree Celsius |
| CTC | Connective tissue cells |
| DHC | Differential hemocyte count |
| DN | Disseminated neoplasia |
| DNA | Deoxyribonucleic acid |
| ELISA | Enzyme-Linked ImmunoSorbent Assay |
| ER | Endoplasmic Reticulum |
| FACS | Fluorescence-activated cell-sorter |
| FITC | Fluorescein isothiocyanate |
| FSC | Forward angle scatter |
| g | gram |
| HN | Hemic Neoplasia |
| hsp | heat shock protein |
| kDa | kilo Dalton |
| kg | kilogram |
| lbs | pounds |
| MA | Massachusetts |
| Mab | Monoclonal antibody |
| ml | millilitre |
| mm | millimetre |
| NADPH | Nicotinamide Adenine Dinucleotide Phosphate-Oxidase |
| NB | New Brunswick |
| NL | Newfoundland |
| nm | nanometre |
| Pab | Polyclonal antibody |
| PAS | Periodic acid Schiff |
| PCB | Polychlorinated biphenyl |
| PE | Prince Edward Island |
| PBS | Phosphate Buffered Saline |
| PI | Propidium iodide |
| RNA | Ribonucleic acid |
| Rb | Retinoblastoma susceptibility protein |
| SE | Standard Error |
| SSC | Side angle scatter |
| TEM | Transmission Electron Microscopy |
| THC | Total hemocyte count |
| µg | microgram |
| µl | microlitre |
| µm | micrometre |
| UPEI | University of Prince Edward Island |
| U.S. | United States |
| QC | Quebec |
| 4N | tetraploid |
| 2N | diploid |

## 1.0 General Introduction

The first documented cases of neoplasia in marine bivalves were described in the 1960's in the American and Pacific oysters, *Crassostrea virginica* and *C. gigas,* respectively, as well as in the blue mussel, *Mytilus edulis* (Farley, 1969 a and b). These conditions were referred to as disseminated neoplasia, and since then, have been described in over 15 bivalve species (Barber, 2004). Two major types of neoplasia are usually recognized in bivalves: gonadal and disseminated (Elston, 1990). Gonadal neoplasia is a progressive disorder involving proliferation of undifferentiated germinal cells. Disseminated neoplasia is a systemic disorder involving the proliferation of cells of unknown origin. Hemic neoplasia (HN) of the soft-shell clam, *Mya arenaria,* is a form of disseminated neoplasia which involves the proliferation of abnormal hemocytes. Since the initial description of hemic neoplasia in the soft-shell clam, there have been many manuscripts and two detailed reviews published (Elston, 1990; Barber, 2004).

Cells described as neoplastic in hemic neoplasia of bivalves are morphologically altered compared to normal hemocytes (Brown et al., 1977; Cooper et al., 1982; Reinisch et al., 1983; Farley et al., 1986; Elston et al., 1992; Barber, 2004). They are described as having a larger nucleus and a higher nucleus to cytoplasm ratio compared to normal hemocytes (Barber, 2004). Neoplastic hemocytes have also been described as lacking the ability to carry out normal functions such as adhesion and phagocytosis (Beckmann et al., 1992). Over the years, researchers studying hemic neoplasia have described the morphology and behavior of these neoplastic cells, yet the biology of normal hemocytes

is still not fully understood. Questions regarding the types of hemocytes, lineages, roles and ontogeny remain. Consequently, the criteria for neoplasia in bivalves are vague and thus when referring to past studies there is some reservation in the diagnosis of neoplasia.

From the point of view of basic immunology, there is interest in studying how hemocyte integrity is altered by neoplasia, as these cells play a fundamental role in immune functions. The soft-shell clam is a very important indicator species used in eco-toxicology studies in Eastern Canada. Indeed, the soft-shell clam is a species of great potential for research, both basic and applied. It adapts well to holding conditions and can be maintained for several months in tanks if proper husbandry is provided. The species is broadly distributed, common, and there are many natural populations in Atlantic Canada. These populations are inter-tidal and sub-tidal in shallow waters, allowing convenient sampling. Adult clams are large, providing sufficient amounts of tissue to allow individual testing. Furthermore, mechanisms in the cellular alterations of hemic neoplasia show this species to be interesting as a human cancer research animal model.

In Atlantic Canada, hemic neoplasia has a negative impact on aquaculture and fisheries (McGladdery and Davidson, 2003). In Prince Edward Island, HN has been a limiting factor in the development of the soft-shell clam aquaculture industry.

### *1.1 Disease description*

Hemic neoplasia is characterized by an increased number of morphologically and functionally altered cells found circulating in the hemolymph and infiltrating tissues (Farley et al., 1986; Elston et al., 1992; Barber, 2004). Descriptions of these altered cells make up the majority of information published on hemic neoplasia. Cytological features are similar in mussels, clams and oysters (Elston et al., 1992). A brief description of the morphology of neoplastic cells will be presented here with a more detailed description in section 1.7. In the soft-shell clam, neoplastic cells are described as being hypertrophied (2-4 times the diameter of normal cells) with a hyperchromatic and pleomorphic nucleus (Barber 2004; Elston et al., 1992). Mitotic figures, especially in advanced stages of the disease, are also observed (Cooper et al., 1982; Farley et al., 1986; Barber, 1990, Brousseau and Baglivo, 1991b). The presence of mitotic figures in advanced stages does not provide confirmatory evidence of neoplasia, as these can be present at any stage of the disease and in normal individuals as well.

Pathological descriptions found in the literature of advanced cases of hemic neoplasia include gill filament and connective tissue disruption (Cooper et al., 1982a) and compression of connective tissue (Brown et al., 1977; Brousseau, 1987). Some authors have described clams with hemic neoplasia as weak animals with pale, watery tissue and mantle recession (Peters, 1988; Smolowitz et al., 1989; Elston, et al., 1992).

Several papers refer to hemic neoplasia as a disorder that renders functional cells non-functional. These non-functional cells are described as overcrowding the healthy cells

resulting in death (Farley, 1986; Cooper et al., 1982(a); Sinderman, 1990; Elston et al., 1992). Hemolymph from neoplastic clams has been described as having a milky appearance due to the large amount of cells (Farley et al., 1986; Brown et al., 1977). Stephens et al. (2001) claimed that clams with hemic neoplasia have a higher hemocyte cell count compared to normal clams.

### *1.1.1 Mortality rate*

Some authors indicate that hemic neoplasia in soft-shell clams can lead to death within 5-6 months from clinical detection (Farley et al., 1986; Cooper et al., 1982(a); Leavitt et al., 1990). Conversely, Appledoorn et al. (1984) reported that clams in a laboratory setting could live up to 3-6 months with advanced cases of hemic neoplasia. Disease progression has been linked to mortality in *Mya arenaria* (Brown et al., 1977; Cooper et al., 1982(a); Brousseau and Baglivo, 1991b). Sunila and Farley (1989) demonstrated that neoplastic cells thrive in hypoxic conditions whereas normal hemocytes die.

A remission phenomenon has been reported in soft-shell clams affected with hemic neoplasia (Barber, 1990; Brousseau and Baglivo, 1991(b)). In a year long field survey, Barber (1990) marked, replanted and sampled 27 hemic neoplasia negative and 27 hemic neoplasia positive soft-shell clams. He found that 15% of clams originally diagnosed with hemic neoplasia by cytology had reverted to a hemic neoplasia negative status. Three of the clams that apparently reverted to hemic neoplasia negative status had an initial disease intensity (percentage of neoplastic cells of total hemocyte numbers) of <2%. The fourth clam which apparently reverted had a disease intensity of

<22.3%. In the same study, 15% of clams originally diagnosed as hemic neoplasia negative were subsequently classified as hemic neoplasia positive with a disease intensity of <4.4 % neoplastic cells. Barber suggested that once clams reach a disease intensity of >23%, remission is not possible (Barber, 1990).

Brousseau and Baglivo (1991(b)) marked and replanted 906 soft-shell clams from Milford Point, Connecticut, to monitor disease progression over a 6-month period. Clams were classified on the percentage of neoplastic cells in the hemolymph as non-neoplastic (<1%), low level (<50%) and advanced (>50%). There was a high mortality rate (48-78%) of clams in the advanced group at the end of the experiment. Complete remission occurred in 16% of the clams initially diagnosed as low-level intensity.

### *1.1.2 Species affected*

Neoplastic disorders in marine bivalves were first described by Farley (1969 a and b) in the American and Pacific oysters, *Crassostrea virginica*, *C. gigas* respectively, as well as in the blue mussel, *Mytilus edulis*. These disorders were termed sarcomatoid proliferative diseases. Since then, similar conditions have been described in over 15 species of marine bivalves worldwide (Barber, 2004). A number of terms have been used in the literature to describe this condition, including haematopoietic neoplasia (Brown et al., 1977; Yevich and Barszcz, 1976; Smolowitz et al., 1989), disseminated sarcoma (Peters, 1988), leukemia (Miosky et al., 1989) and hemic neoplasia (Reinisch et al., 1983). The variability of terminology stems from differences in interpretation and lack of consensus in mollusc pathology. Elston et al. (1992) suggested that the term

disseminated neoplasia should be used to avoid false assumptions of which progenitor cell or cells are involved. In the soft-shell clam, *Mya arenaria*, this disorder has been specifically referred to as hemic neoplasia (Reinisch et al., 1983).

Cases of disseminated neoplasia have been documented in a range of marine bivalves including: the blue mussel, *Mytilus edulis* in Denmark (Rasmussen, 1986), England (Lowe and Moore, 1978), and Finland (Sunila, 1989), the common cockle, *Cerastoderma edule* in Ireland (Twomey and Mulcahy, 1988) and France (Poder and Auffret, 1986), the European flat oyster, *Ostrea edulis,* in Spain and Yugoslavia (Alderman et al., 1977) and the truncated mussel, *Mytilus truncata* in Canada (Neff et al., 1987) and *Cerastoderma edule* in Spain (Villalba et al., 2001). As well, hemic neoplasia has been extensively studied in *Mya arenaria* during the 1970's and 1980's along the eastern U.S. coast from Florida to Maine (Barber, 2004). In Canada, the majority of studies are from Prince Edward Island. Outbreaks of hemic neoplasia in soft-shell clams in Eastern Canada have been documented by McGladdery et al. (2001). Morrison et al. (1993) reported hemic neoplasia in 6/22 sites in New Brunswick and Nova Scotia, in a two-year histological study documenting prevalence of 3.1- 31%. McGladdery et al. (2001) reported a prevalence of 95% in one area in Prince Edward Island in a histological survey. This was the first case of mass mortalities associated with hemic neoplasia in Atlantic Canada. McGladdery et al. (2001) conducted a small-scale geographical survey reporting hemic neoplasia prevalence in sites in New Brunswick (15-19%), Nova Scotia (0-0.3%), Newfoundland (0%) and Prince Edward Island (23-29%).

## 1.2 Effect on fishery and aquaculture

As a result of mortalities associated with hemic neoplasia, there have been negative impacts on both wild and cultured soft-shell clams. Historically, the soft-shell clam has had a long and prosperous record in the North American seafood market. The average price has risen from $2.10/kg in 1995 to $3.10/kg in 2000 (McGladdery et al., 2001). In 1999, the commercial landed value of soft-shell clams in the Gulf of St. Lawrence was 1065 metric tons (2.27 million lbs) valued at $ 2.46 million. In 1999 and 2000 on Prince Edward Island, 986 metric tons was the commercial landed weight of soft-shell clams (PEI Agriculture, Fisheries and Aquaculture, 2006). Due to a high prevalence of hemic neoplasia in 1999, the soft-shell clam industry has struggled. Setbacks associated with hemic neoplasia not only have negative effects on the aquaculture industry, but on commercial and recreational fisheries as well. Digging for soft-shell clams in Atlantic Canada has provided seasonal employment and a source of income for many coastal communities. As well, it has been a favorite pastime for locals and tourists.

## 1.3 Disease occurrence

Seasonal trends have been reported in most areas where hemic neoplasia has been found in soft-shell clams. Appledoorn et al. (1984) reported year round prevalence of hemic neoplasia in Washington DC, with the highest peak prevalence reported in the fall and late spring. These authors suggested that low temperature could suppress the progression of hemic neoplasia. Metabolic activity may slow down during these winter months and the clams may not expend as much energy. On the contrary, during the summer the clams allocate energy for gametogensis, leaving them more susceptible to

secondary stressors. Leavitt et al. (1990) reported that the highest prevalence of hemic neoplasia (39.3%) occurred during the fall (September-October) in Little Buttermilk Bay, Massachusetts. In a multi-year study in Long Island Sound, Brousseau (1987) reported the highest prevalence of hemic neoplasia in clams during the late fall and early winter and the lowest prevalence during late spring to early summer. This pattern was similar to that observed for *Mytilus edulis* from Yaquina Bay, Oregon (Mix 1983) and from Lillebaelt, Denmark (Rasmussen, 1986).

Barber (1990) reported that hemic neoplasia prevalence was negatively associated with individual clam disease intensity. This year-long New Jersey study reported a maximum mean intensity in October and April while hemic neoplasia prevalence was the lowest. As intensity decreased, prevalence increased in December and May. In Canada, McGladdery et al. (2001) reported a lack of correlation between hemic neoplasia and season in clams from the Gulf of St. Lawrence and Bay of Fundy area. In the U.S., it appears that hemic neoplasia can occur year round but seems more prevalent in winter months (Barber, 1990). The use of different staging criteria and diagnostic methods make it difficult to observe direct comparisons of the seasonal patterns from all of these studies. Subjectivity of the researcher and the sensitivity of the diagnostic tool applied can lead to differing results.

## 1.4 Causation

The etiology of hemic neoplasia is believed to be multi-factorial. Numerous studies have attempted to link a possible etiology for a given time and area. Possible etiologies causing hemic neoplasia include: (1) anthropic and (2) viral.

### *1.4.1 Anthropic influences*

Several studies have attempted to address the issue of a possible correlation between hemic neoplasia and exposure to anthropic activities. Terrestrial and marine anthropic activities put pressure on coastal environments through urbanization, industrial activities and transportation. This pressure can be the result of the source, the pollutant and the type of impact. Pollutants such as polychlorinated biphenyls, polynuclear aromatic hydrocarbons, petroleum fuels, herbicides, heavy metals and domestic sewage can be harmful to the well being of marine animals. Eco-toxicological studies using bivalves as bio-indicators of the ecosystem integrity are very common (Pipe et al., 1995).

Strandberg et al. (1998) documented a correlation of specific reactivity of tissues with polychlorinated biphenyl (PCB) antibodies in soft-shell clams from one contaminated and two non -contaminated sites in Maine. There was a high prevalence of hemic neoplasia at contaminated sites and a low prevalence at non-contaminated sites. In this study, 1E10 (monoclonal antibody for neoplastic hemocytes) antibody was used to detect neoplastic hemocytes. Polychlorinated biphenyls were found in normal and neoplastic hemocytes in the gills, ovaries and digestive gland. Reactivity with the

polyclonal antibody to PCB was associated with a high prevalence of hemic neoplasia in areas of high pollution. Yevich and Barszcz (1976) reported high levels of disseminated neoplasia in soft-shell clams from an oil spill site in Searsport Bay, U.S.

Not all reports are supportive of a pollutant etiology. Appledoorn et al. (1984) sampled soft-shell clams from sites polluted with varying degrees of sewage, heavy metals and oil spills in northeastern U.S.A. and Canada, and found no correlation between the prevalence of hemic neoplasia and the degree of pollution. Failing to find a correlation does not rule out the possibility of past pollutant effects at the site. Multiple time frame analysis is required. Twomey and Mulcahy (1988(a)) surveyed 19 locations in Ireland and found that disseminated neoplasia was found in the common cockle, *Cerastoderma edule*, in sites that were impacted with heavy metals including copper, lead and cadmium, as well as in sites that were free of pollutants. There was no significant difference in the level of prevalence in the contaminated and non-contaminated sites. McGladdery et al. (2001) conducted a two-year laboratory and field study in response to high mortalities of soft-shell clams in Prince Edward Island. These clams were sampled from beds situated near agricultural sites. Clams were exposed to chlorothalonil, a commonly used fungicide, for 27 days at a dose of 1-1000 μg/l. Exposed clams did not develop hemic neoplasia.

In a recent study (Pariseau et al., in prep) exposed 80 Prince Edward Island hemic neoplasia negative clams to commonly used fungicides. The experiment took place over

a period of 6 weeks and clams were exposed at various concentrations of fungicide. The results revealed no induction of hemic neoplasia.

There is evidence that contaminants can compromise the immune system of bivalves, thus leaving them susceptible to secondary stressors (Pipe et al., 1997). It is important to recognize that anthropic, geographical and environmental differences exist and may influence the amount of pressure placed on the ecosystem. Thus, the situation in one area may be entirely different from another. For example, in New Bedford Harbor, Massachusetts, the prevalence of hemic neoplasia in soft-shell clams is consistently high (22-70%) and disease intensity is very high (Reinisch et al., 1983; Leavitt et al., 1990). This harbor is heavily influenced by anthropic activities contributing to high levels of PCBs in both the sediments and water column (Strandberg et al., 1998).

### *1.4.2 Viral influences*

Alterations of the normal cellular checks and balances contribute to normal cells becoming neoplastic over time. These include growth promoting proto-oncogenes, growth inhibiting suppressor genes, regulatory genes and DNA repair genes. Normal genes can become altered spontaneously or through exposure to mutagenic agents. Neoplastic cells often contain several mutated genes including those involved in the normal progression of the cell cycle. These cells have lost the ability to be controlled and do not require external growth signals to grow. For example, proto-oncogenes regulate normal cell growth and differentiation. Proteins encoded by these genes function as growth factor ligands and receptors, signal transducers, transcription factors

and cell cycle components (Kumar et al., 2005). Altered expression or mutations of proto-oncogenes can cause them to switch to cellular oncogenes (c-oncs) involved in tumor development (Kumar et al., 2005). Oncogenes promote uncontrolled growth in neoplastic cells. Proto-oncogenes turn into oncogenes due to changes in the gene structure and gene expression. These changes arise from point mutations, chromosomal re-arrangements and gene amplification (Cockerall and Cooper, 2005). For malignant tumors to develop, several proto- oncogenes usually must be altered. Oncogenes can have negative affects on various stages of normal cell replication and development. Viral oncogenes can arise from DNA and RNA viruses. Oncogenic DNA tumor viruses such as herpes viruses may inactivate some of the host tumor suppressor genes or enhance the activity of growth factors (Kumar et al., 2005). This basically causes a stimulation of the host cell containing virus to move into the S phase while suppressing apoptosis in order to replicate. Retroviruses have an oncogenic RNA genome and are divided into acute and chronic transforming forms.

Studies with hemic neoplasia have suggested that the etiology is due to an infectious agent, possibly a retrovirus. Oprandy et al. (1981) purified viral particles of 120 nm from neoplastic hemocytes of the soft-shell clam and morphology as a retrovirus was confirmed by electron microscopy. Purified virus was inoculated into clams without neoplasia, which then developed hemic neoplasia within 2 months. McLaughlin et al. (1992) and House et al. (1998) criticized this study because of a lack in reproducibility. Although Oprandy confirmed the presence of viral particles using electron microscopy,

McLaughlin et al. (1992) and House et al. (1998) failed to find viral particles using electron microscopy in similar studies.

Studies have demonstrated that hemic neoplasia can be transmitted by transplantation of whole cells from bivalve to bivalve. Questions remain as to whether this is a case of transplantation, whereby the donor neoplastic cells proliferate, or true transmission, involving an agent viral released from the donor cells. Cell free transmission attempts have failed to cause hemic neoplasia in the soft-shell clam. McLaughlin et al. (1992) examined the possibility of transmission with the use of a cell free ultrafiltrate. One group of healthy clams was inoculated with unfiltered hemolymph and a second group was inoculated with cell free ultrafiltrate previously sonicated and filtered. The group inoculated with unfiltered hemolymph developed neoplasia whereas the clams inoculated with ultrafiltrate failed to develop neoplasia. House et al. (1998) examined this further by injecting healthy clams with whole neoplastic cells, unfiltered homogenate and filtered cell homogenate. These authors found that only clams injected with whole cells developed neoplasia. Evidence of reverse transcriptase was also observed in clams with advanced levels of neoplasia. Reverse transcriptase alone is not enough to conclude that a retrovirus is the cause of hemic neoplasia without evidence of molecular weight, budding and detection of viral particles by negative staining. The latter two are determined by electron microscopy.

### *1.4.3 Environmental influences*

The trigger(s) for hemic neoplasia may differ geographically and could be influenced largely by the integrity of the ecosystem. It is quite remarkable how bivalves conform to their changing environment on a regular basis. However, multiple stressors can play a role in altering the integrity of these animals, which ultimately could promote the onset or progression of hemic neoplasia. Most studies neglect to include environmental variables (temperature, salinity, sediment type, food availability or pathogens) that can affect the physiology of the animal. These factors, including age, can play a role in the health status of bivalves. Changes in water temperature and salinity (Leavitt et al., 1990) as well as overcrowding (Ford et al., 1994) have been suggested to influence hemic neoplasia prevalence in some areas. Quantitative data are still needed to confirm this.

### *1.4.4 Genetic influences*

Genetics can play a role in susceptibility to cancer in humans (Kumar et al., 2005). Alterations and loss of tumor suppressor genes or activation of oncogenes can be inherited. Determining if clam populations are from the same family or are genetically different could provide useful information on whether or not populations are more susceptible to hemic neoplasia. Seed from less susceptible populations could be used for culturing or restocking purposes. Studies have attempted to show that the overexpression of mutant p53 or mutational inactivation of the p53 tumor suppressor gene in the soft-shell clams leads to neoplasia. The p53 protein plays a very significant role in preventing uncontrolled cell growth. It acts as a transcriptional activator,

controlling the activation of genes involved in cell cycle regulation and apoptosis. Alteration of this gene contributes to more than half of human cancers (Kumar et al., 2005). Barker et al. (1997) investigated normal and neoplastic hemocytes from soft-shell clams in New Bedford Harbor and Chatham, Massachusetts with the use of a murine polyclonal antibody termed Pab 240 which is reactive for mutant p53. Clams were sampled from a positive and negative site, diagnosed as positive or negative with 1E10 antibody specific for neoplastic cells, then exposed to Pab 240. Neoplastic cells displaying a positive reactivity by immunofluorescence by Pab 240 were evaluated as well as controls. The authors described a mutation in the central DNA binding site that caused a substitution in the first part of the codon resulting from the shift of proline to alanine. The Pab240 identified the mutated p53 gene at this site. Only clams diagnosed as having an "advanced" stage of hemic neoplasia were positive using the antibody. This study suggests that mutant p53 expression may play a role in hemic neoplasia, but uncertainties remain. A very small sample size of 5 clams with hemic neoplasia were used and only 2/5 clams were shown to have this mutation. There was a lack of correspondence between the mutation of the gene and the p53 sequence. The possibility of a mutation in the p53 gene involved in hemic neoplasia in clams has not been repeated since this study was published.

Kelley et al. (2001) utilized homologies of human p53, such as p73, for corresponding protein analysis in tissue from clams with and without hemic neoplasia. The p73 was determined to be larger than p53 and upregulated in both neoplastic hemocytes and adductor muscle. Both p53 and p73 were sequestered outside the nucleus. This

cytoplasmic sequestration may lead to inhibition of antiproliferative activities that in turn could promote the proliferation of cancer cells due to the inability of p53 to act in the nucleus. Cytoplasmic sequestration of p53 is common in human cancer (Kumar et al., 2005; Walker et al., 2006). Walker et al. (2006) suggested that when p53 protein is over expressed in soft-shell clams, it becomes trapped outside of the nucleus due to mortalin. In this sense, mortalin acts like an anchor protein, binding to the cytoplasmic domain of p53 and trapping it outside the nucleus (Kaul et al., 2002). This interaction sequesters p53 in the cytoplasm preventing nuclear translocation (Dundas et al., 2005). Mortalin is a member of heat shock protein 70 (hsp70) that resides in the endoplasmic reticulum, mitochondria, plasma membrane, cytoplasmic vesicles and cytosol (Kaul et al., 2002). It is involved in multiple functions including stress response, control of cell cycle proliferation, differentiation, and tumorigenesis (Wadhwa et al., 2002).

### 1.5 Diagnosis of hemic neoplasia

Traditional methods for diagnosing hemic neoplasia are histopathology and hemocytology. Researchers have adopted similar protocols from earlier descriptions by Farley (1969 a and b) for hemocytology. The stain used most often is Feulgen picro-methyl blue. Disease stages differ between authors for hemocytology and histopathology (Leavitt et al., 1990; Mix, 1983; McGladdery and Davidson, 2003).

With histopathology, the intensity is based on the tissue infiltration severity throughout the body (Mix, 1983). For example, Mix (1983) assigned stages of disease severity for mussels Stage 1: small foci of neoplastic cells, Stage 2: neoplastic cells begin to

infiltrate connective tissue, Stage 3: neoplastic cells in the sinus, Stage 4: total body burden of neoplastic cells. Histology provides information on the tissue architecture and location of neoplastic cells in the body. Histology is a lethal test and is time demanding and expensive, which limits its use in some studies. Additionally, low- level cases may go undetected due to the small 3-4 μm sections that are processed for each individual. For hemocytology, disease intensity is rated as the percentage of neoplastic cells in the total number of hemocytes examined (Farley et al., 1986). This method involves the withdrawal of approximately 0.1 - 0.5 ml of hemolymph from the adductor muscle or pericardial cavity. Samples are placed on a glass slide and examined with phase contrast optics or stained. Various scales have been developed over the years for hemocytological analysis (Table 1).

Hemocytology offers a non-lethal assay that provides a visual examination of the hemocytes. Disease progression, on an individual level, can be followed temporally with disease severity determined in terms of the percentage of abnormal cells. Both histopathology and hemocytology rely on morphological characteristics, which can be subjective in nature and problematic due to the lack of information regarding normal hemocytes. However, both methods qualitatively provide a framework of reference for comparing normal and abnormal conditions and disease progression.

Immunohistochemistry is another method used in the diagnosis of hemic neoplasia. Reinisch et al. (1983) and Smolowitz and Reinisch (1986) developed an indirect immunochemical technique in conjunction with a monoclonal antibody (1E7) specific

for neoplastic cells in the soft-shell clam. Following labeled antibody attachment, fluorescence was detected on the neoplastic cells in contrast to normal cells. As well, an unspecified cell was also identified by this antibody, which the authors refer to as a neoplastic precursor cell. From the same group of investigators, Miosky et al. (1989) reported a sister monoclonal antibody of 1E7, termed 1E10; this antibody was claimed to be specific for soft-shell clam neoplastic hemocytes. This antibody identified a specific protein on the surface of cells in both tissue sections and hemolymph samples. A few years later, White et al. (1993) developed an antibody (2A4) specific to normal hemocytes in the soft-shell clam.

This antibody was utilized using an Enzyme-Linked ImmunoSorbent Assay (ELISA), immunohistochemistry, Western blotting and flow cytometry. As hemocytes in an advanced stage of hemic neoplasia may lose 2A4 reactivity, this antibody may be useful in tracking disease progression. Similarly, Stephens et al. (2001) identified a 252-kDa-protein specific to neoplastic cells, which reacted with the 1E10 antibody and a 185-kDa protein specific to normal cells, which reacted with 2A4. The 2A4 reacted with both normal cells and a subset of neoplastic cells that the authors referred to as transitional cells (Stephens et al., 2001). These transitional cells apparently retain the ability to adhere to a glass surface unlike transformed cells in advanced cases of hemic neoplasia; they could be the same cells that Smolowitz and Reinisch (1986) described.

**Table 1**: Hemocytological scales employed in the past to evaluate hemic neoplasia progression in the soft-shell clam.

| Example | Stages | % Neoplastic cells |
|---|---|---|
| Farley et al. (1986) | Early | 0.01 – 0.9% |
| | Intermediate | 1 – 49% |
| | Advanced | 50 – 100% |
| Leavitt et al. (1990) & White et al. (1993) | 1 | <15% |
| | 2 | 16 – 70% |
| | 3 | >71% |
| Brousseau & Baglivo (1991a) | Low Level | 1 – 50% |
| | Advanced | 51 – 100% |

Effective detection and assessment of hemic neoplasia requires the use of techniques that are non-lethal, labor efficient, low- cost, reliable and able to assess large numbers of individuals in a short period. Flow cytometry is a technique for counting, examining and sorting cells. Flow cytometry allows the simultaneous multiparametric analysis of the physical and chemical characteristics of single cells as they rapidly move through a tubular flow system (Ashton-Alcox and Ford, 1998). Flow cytometry has demonstrated its potential for investigating bivalve hemocyte populations, activities, functions (Ashton-Alcox and Ford, 1998; Xue et al., 2001; Delaporte et al., 2003; Hegaret et al., 2003) and disorders such as disseminated neoplasia (Elston et al., 1990; White et al., 1993; Reno et al., 1994).

Flow cytometry provides the ability to objectively screen large numbers of cells, approximately 3000 cells/sample in a few seconds, which greatly exceeds the capacity of traditional methods such as cytology or histology. It also provides an objective method for analyzing DNA ploidy in hemocytes. Flow cytometry was used in several studies for evaluating the DNA content in normal and neoplastic cells in a number of mollusc species (Reno et al., 1994; Da silva et al., 2005). The protocol most commonly used to detect hemic neoplasia is based on DNA quantification with the use of fluorescent dye as propidium iodide (Reno et al., 1994; Da Silva et al., 2005; Smorlarz et al., 2005a).

## 1.6 Bivalve hemocytes

### *1.6.1 Ontogeny and classification*

Currently, the most important reviews of the various morpho-functional aspects of molluscan hemocytes are those of Cheng (1981) and Hine (1999). It is agreed that the two main cell types are granulocytes and agranulocytes (Cheng, 1981, 1984; Fisher, 1986; Auffret, 1988; Cheng, 1996; Chu, 2000). Agranulocytes are also referred to as hyalinocytes. The presence of these two cell types have been confirmed in *Mya arenaria* (Huffman and Trip, 1982; Seiler and Morse, 1988), *Mercenaria mercenaria* (Allam et al., 2002), *Ruditapes decussatus* (Lopez et al., 1997a), *R. philippinarum* (Allam et al., 2002), *Mytilus edulis* (Pipe, 1997; Noel et al., 1994; Wooton et al., 2003), *M. galloprovincialis* (Carballal et al., 1997), *Crassostrea virgnica, C. gigas* (Ford et al., 1994; Cheng, 1981; Ford, 1994; Allam et al., 2002; Ashton-Alcox and Ford, 1998) and *Cerastoderma edule* (Wooton et al., 2003). The process of categorizing hemocyte types is tedious and variations occur because of subjectivity, sampling, environment, age, physiology and methodology. Variations also occur because bivalves and their hemocytes can vary considerably from one another. Distinguishing between granular and agranular cells can be problematic. For example, a cell appears that has few granules but lacks the other characteristics of granular cells can be difficult to categorize as granular or agranular. The classification of bivalve hemocytes has been a topic of interest and/or debate for many years. Cell classification and ontogeny is a controversial subject in bivalve immunity as the site of hematopoesis and the cellular maturation stages remain unknown. Mix (1976) proposed the one cell theory whereby hyalinocytes represent a proliferative stage that become granulocytes when mature

(Figure 1). In contrast, Cheng (1981) suggested that granulocytes and agranulocytes represent two distinct cell lines, and that their developmental stages account for the subpopulations of cells described by others. He also included serous or brown cells as a third cell line (Figure 2).

Whether developmental stages occur between immature and mature circulating cells is not known. There is general agreement that the hemocytes arise from differentiation of connective tissue cells (Mix, 1976; Cheng, 1984), and that the division of blast cells (defined as immature, undifferentiated precursor cells) occurs in the hemolymph (Hine, 1999). It is possible, however, that differentiated cells may retain the ability to divide (Hine, 1999). Smolowitz et al. (1989) demonstrated the binding of monoclonal antibody (MaB) 4A9 to three cells: neoplastic cells, a subset of normal circulating cells and connective tissue cells (CTC) lining the hemolymph sinuses of *Mya arenaria*. The authors suggested that the CTC may be the cell of origin of both the normal hemocytes and neoplastic cells. They further suggested that the subset of normal looking hemocytes may be a transforming stage of neoplastic cells. Further criteria for determining normal from neoplastic cells is required. Morphological characteristics of neoplastic cells will be discussed in the next section. Emphasis on the functional roles of these cells are highlighted in section 1.8.

### *1.6.2 Granular hemocytes*

Granular cells have been reported in most bivalves including; mussels, oysters, clams, and cockles (Hine, 1999). Scallops are an exception as they have only hyalinocyte type

of hemocyte (Auffret, 1988). Granulocytes usually have a low nucleus to cytoplasmic (N:C) ratio and display a spherical to ovoid eccentric nucleus (Hine, 1999). They form pseudopodia referred to as filopodial (spikey) projections. These cells are described as active cells involved in phagocytosis and clumping (Cheng and Howland, 1979, 1981; Cima et al., 2000). Granular cells vary in size and tinctorial properties. Based on tinctorial characteristics of their granules, granulocytes can be further divided into basophilic and eosinophilic subcategories. Some researchers divide granulocytes into finely granular or coarsely granular sub-categories based on the number of granules present in the cytoplasm (Cheng, 1981, 1984; Hine, 1999).

Some granulocytes have more than one type of membrane bound granule (Hine, 1999). Transmission electron studies have shown that hemocyte granules vary in size from small to large and can be electron dense or electron lucent. These granules contain different enzymes including esterases, $\beta$-glucuronidase and peroxidase (Chu, 2000). Some granules contain acid hydrolases and may be considered a form of lysosome (Lopez et al., 1997b). Granulocytes have been shown to phagocytose bacteria, heat killed yeast, algae, cellular debris, carbon particles, parasites and latex beads (Cheng, 1996; Caraballal et al., 1997; Hine, 1999; Chu 2000). In *Mytilus spp, Crassostrea virginica, C. gigas* and *Mya arenaria,* granulocytes are more active in phagocytosis than agranulocytes (Lopez et al., 1997b). Wooten et al. (2003) described equivalent phagocytic ability of both granulocytes and agranulocytes of the clam, *Ruditapes phillipinarum.*

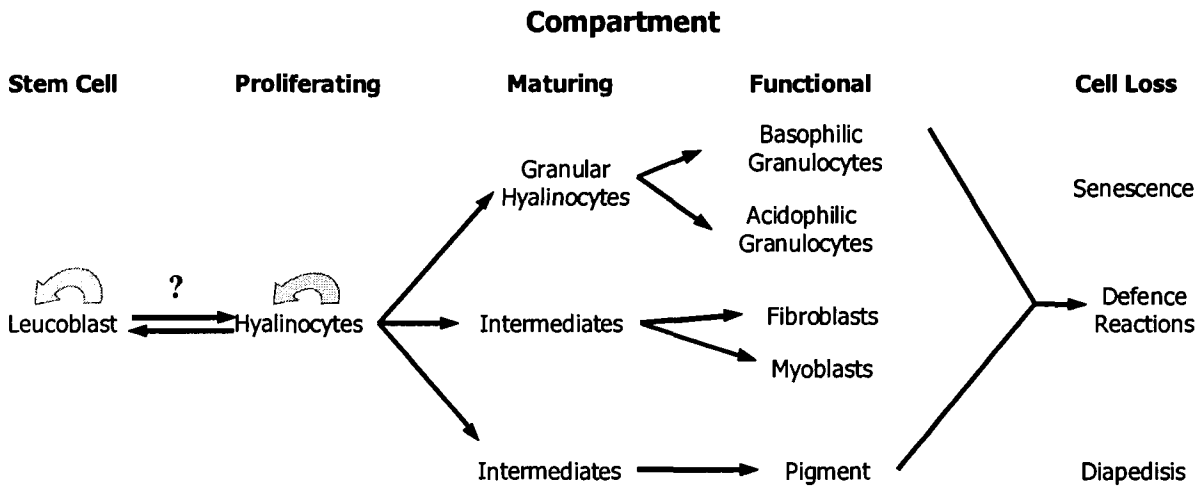


**Figure 1**: Mix (1976) one cell theory of bivalve cells

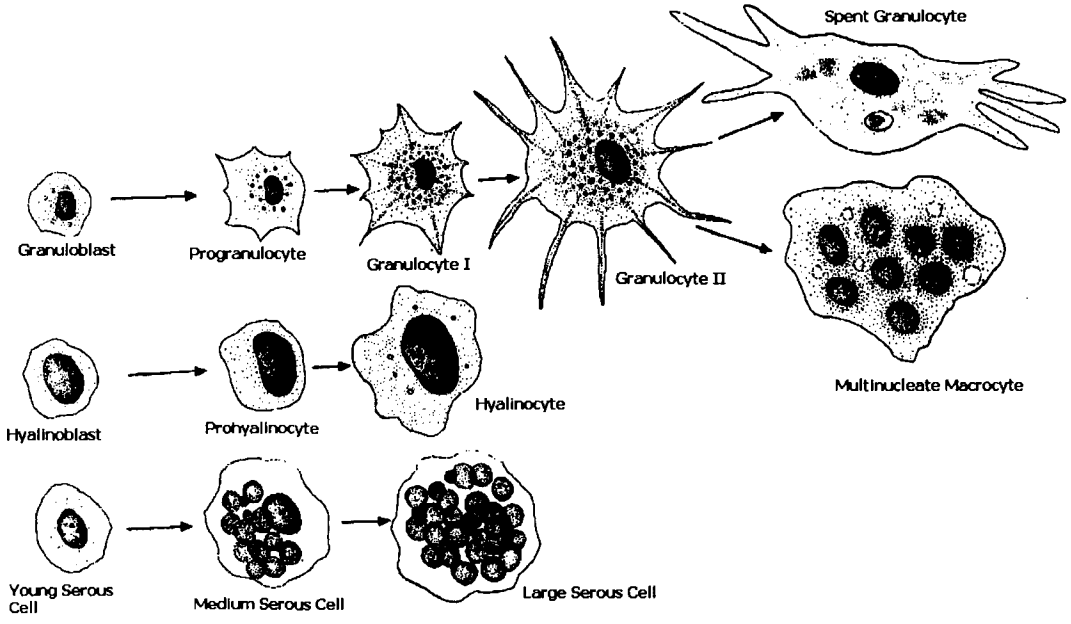


**Figure 2**: Cheng (1981) hypothetical lineage of bivalve cells

### *1.6.3 Agranular hemocytes*

Agranular hemocytes either have no or very few cytoplasmic granules. Some refer to these cells as hyalinocytes (Hine, 1999; Allam et al., 2002). Based on Hine's review, three categories of agranular cells can be considered in bivalve molluscs: blast-like cells, macrophage-like cells with basophilic cytoplasm and hyalinocytes. Agranular cell pseudopodial projections have been described as lobopodial (round in shape) and the cells have been demonstrated *in vitro* to move slower than granulocytes. Blast-like cells are characterized by their central ovoid nucleus and high N:C ratio. Macrophage-like cells are characterized by their large, eccentric nuclei and copious amounts of cytoplasm (Hine, 1999). Hyalinocytes are characterized by their round to ovoid nucleus, low N:C ratio and light blue cytoplasm. Information is lacking on agranular cell functional capabilities. Some authors have reported agranular cells as the most active cells involved in phagocytosis (Allam et al., 2002) while others disagree (Hine, 1999).

### *1.6.4 Ceroid cells*

Ceroid cells are also referred to as serous, brown, pigmented or rhogocyte cells. These cells are a brown color due to a wax - like substance that accumulates when there is a disruption of fat metabolism (McGladdery et al., 2001). Some researchers believe they arise from the pericardial gland and belong to a different developmental pathway than hemocytes. However, their relationship to hemocytes is unknown. According to Cheng (1981) and Hine (1999), ceroid cells are phagocytes that are found usually in the reno-pericardial region. They are commonly seen in molluscs subjected to pollution, but these cells are also found to a lesser degree in healthy animals.

The categorization of bivalve immune cells to date has been largely based on differential staining, lectin binding and enzymatic studies. With regard to clam species, there are only a few studies highlighting hemocyte characteristics. The majority of these studies had observations based on morphological and cytoenzymatic methods. These included studies of *Mya arenaria* (Huffman and Trip, 1982), *Tapes philippinarum* (Cima et al., 2000) and *Chamelea gallina* (Pampanin et al., 2002). The work to be described in this thesis used light microscopy and ultrastructural morphology to describe hemocytes of *Mya arenaria.*

Flow cytometry has also been used to elucidate the cell categories for the oyster, *Crassostrea virginica, C. gigas, Ostrea edulis,* the common cockle, *Cerastoderma edule* (Ford et al., 1994; Xue et al., 2001; Ashton-Alcox and Ford, 1998) and scallop, *Pecten maximus* (Auffret, 1988). Other authors have used flow cytometry coupled with light microscopy on *Ruditapes philippinarum* and *Mercenaria mercenaria* (Allam et al., 2002).

## 1. 7 Characterization of Neoplastic cells

### *1.7.1 Cell ontogeny*

The origin of neoplastic cells in bivalves is largely unknown. These abnormal cells are poorly differentiated, making it impossible to tell the tissue of origin. Bivalves have an open circulatory system, making it difficult to document tissue invasion because hemolymph is in contact with every tissue. Several authors support the idea that the neoplastic cells in hemic neoplasia are hemocytic in origin although confirmation is not

possible because of the lack of knowledge on bivalve hematopoiesis (Elston, 1990). Neoplastic cells resembling hyalinocytes seem to appear first in the vascular spaces of the soft-shell clam (Farley 1970; Balouet and Poder, 1979). Cooper (1982) described transitional neoplastic cells resembling hemocytes in the mussel, *Mytilus edulis*. Balouet et al. (1979) described neoplastic cells in *Ostrea edulis* as having strong morphological similarities with normal hyalinocytes. Elston et al. (1988) noted transitional forms of neoplastic cells in mussels resembling normal hemocytes in morphology and behavior.

Immunocytological evidence suggests that the neoplastic cells in hemic neoplasia are hemocytes (Reinisch et al., 1983; Smolowitz et al., 1989). To determine if unique antigens are found on neoplastic cells in the soft-shell clam, Reinisch et al. (1983) produced 10 antibodies. Only one reacted with both neoplastic cells and hemocytes and the other nine reacted with only neoplastic cells. The authors suggested that the antibodies failed to cross- react because normal cells arise from different tissue origins. This paper does not provide information on controls or sample number. In another study, Smolowitz et al. (1989) evaluated clams with the use of Mab 1E10 and Mab 4A9 to determine disease intensity. The latter antibody reacted with neoplastic cells, normal cells and a small population of connective tissue cells. The authors used this information to suggest that connective tissue cells are the cells of origin for both neoplastic and normal hemocytes. Based on their morphological description, these cells do not seem like two different cell types but may be neoplastic cells at different transitional stages. Using antibodies to identify the tissues that cross-react or share

common epitopes with the neoplastic cells has been a proven success in vertebrate systems. Information on the embryological and developmental origin of hemic neoplastic cells is required. Further work with Mab 4A9 with a greater focus on the morphological description of hemocytes identified by this antibody could be beneficial for cell ontogeny studies.

### *1.7.2 Neoplastic cell morphology*

Cytological changes characteristic of neoplastic cells have been well documented in several species of bivalves. Based on morphology, neoplastic cells in the soft-shell clam are described as large round cells approximately 10-20 μm in diameter (Miosky et al., 1989; Moore et al., 1992) with a high N:C ratio containing one or more nucleoli (Yevich and Barszcz, 1976, 1977). Through flow cytometric and chromosome analysis, Reno et al. (1994) discovered that neoplastic cells have 1.25 - 2.05 times more DNA than normal cells and a chromosome number ranging from 44-80 compared to 26-39 normally. Neoplastic cells have been described as losing their ability to adhere to substrates and perform phagocytosis. Beckman et al. (1992) conducted a comparative study on the phagocytic ability of normal and neoplastic hemocytes. In this study, neoplastic cells were unable to adhere or ingest yeast cells due to the inability to extend pseudopodia. However, the number of clams used in this study was small. As the phagocytic ability of hemocytes is greatly influenced by season, this factor should be taken into consideration when interpreting results of this study.

Neoplastic cell ontogeny in bivalves has been investigated. Some researchers have described a single cell lineage (clonal hypothesis) whereby a single morphologically transformed cell replicates and produces other fully transformed cells. According to Brown et al. (1971), two types of neoplastic cells have been described in the soft-shell clam. Type 1 cells are described as large, anaplastic appearing cells with little cytoplasm, multiple nucleoli and frequent mitotic figures. Type 2 cells are described as round, intermediate sized cells with one prominent nucleoli. Both types were found together and separately in the 69 clams sampled in the study (Brown et al., 1971). Cellular studies on the soft-shell clam are limited, so information is lacking on the cellular changes that occur during the course of hemic neoplasia. Farley and Sparks (1970) used cytology to describe two types of cells involved in proliferative disorders thought to be neoplasia in *Crassostrea virginica* and *Mytilus edulis*. Enlarged cells with hypertrophied nuclei characterized the first type of presumed neoplastic cell. Other features included pleomorphism, mitotic figures and binucleation. The second type of presumed neoplastic cell was described as having a round shape and a uniform nucleus with large amounts of RNA in the cytoplasm. The authors describe presence of the latter cell type as the hyaline hemocytic type disorder. A description of the morphology of normal hyaline cells was not provided in that study. Lowe and Moore (1978) described a proliferative disorder consisting of two types of hemocyte-like presumed neoplastic cells in *Mytilus edulis* from United Kingdom. Histology was performed on 994 mussels and 16 were determined to be neoplastic. Examination of hemolymph smears stained with Feulgen stain in this study revealed two types of presumed neoplastic cells. The authors describe the two types of cells as being similar to normal

basophilic agranular hemocytes. The first abnormal cell, Type A, was characterized as having a diameter of 8-9 μm with an irregular lobed nucleus, diffuse chromatin and multiple nucleoli. The type B cell was described as having a 9-10 μm diameter, a 6-7μm nucleus with a single nucleolus and dense chromatin. Type A was found more frequently than Type B. Scanning microdensitometry of Feulgen stained nuclei revealed a higher DNA value in the two abnormal cell types compared to normal hemocytes. A morphologically intermediate stage between the two types was observed, suggesting the possibility of a single cell lineage. Green and Alderman (1983) evaluated 4000 blue mussels from the United Kingdom with cytology. Examination of the cells revealed two types of abnormal cells, which corresponded with those described by Lowe and Moore (1978). However, transmission electron microscopic analysis of a small sample (size not provided) revealed only one type A cell. It was described as having a grossly enlarged ovoid nucleus, clumped chromatin, nuclear invaginations and many vacuoles in the thin rim of cytoplasm. In scanning electron microscopy, normal cells attached to the substrate with extended pseudopodia whereas presumed neoplastic cells (type not specified) did not. As in Lowe and Moore's work (1978), the authors do not provide the stage of disease or the criteria used to diagnose the disease. As well, their sample size was very small. Whether or not these cells are believed to be from a single cell line was not alluded to by the authors.

Moore et al. (1991) described the co-existence of two presumed neoplastic cell types based on flow cytometric analysis in blue mussels, *Mytilus edulis.* This study revealed variations of DNA content in the presumed neoplastic cells of 73 mussels. Cells

containing 3.8 x haploid (tetraploid) and 4.9 x haploid (pentaploid) were assumed to represent two distinct forms rather than intermediate stages of a single process. Tetraploid forms were characterized as smaller cells with less cytoplasm, double nucleoli and more mitotic figures. A description of the pentaploid forms was absent. The authors suggest that these two cell forms represent separate processes and reflect cell variation found in the cell cycle. Descriptions of morphological alterations of the two presumed neoplastic cells seem to be similar in several bivalve species. It is possible that these two types originated from one cell, which underwent multiple mutations into different morphological variants. It is still also possible that two cell lineages are involved. All of these ideas are speculative and warrant further work.

In light of the previous information, the purpose of the next section on bivalve immunity is two-fold. First, it is meant to offer readers an overview of available information. Second, it will emphasize the important role of hemocytes and what alterations can occur if their functions are altered and/or lost due to hemic neoplasia.

## 1. 8 Immune systems of Molluscs

### *1.8.1 Brief overview of innate immunity*

There are over 100,000 species of bivalve molluscs identified (Hine, 1999). Despite this massive diversity, their immunology has only been studied in a few economically important species. These include marine mussels, *Mytilus* spp. (Pipe, 1997; Noel et al., 1993; Mitta et al., 1999; Mitta et al., 2000; Pruzzo et al., 2005), the American oyster, *Crassostrea virginica* (Rodrick and Cheng, 1974; Cheng, 1981, 1984), the clam,

*Ruditapes* spp. (Lopez et al., 1997a,b; Allam et al., 2000; Ordas et al., 2000; Tafalla et al., 2003), the soft-shell clam, *Mya arenaria* (Rodrick, 1979; Blaise et al., 2002) and the hard clam, *Mercenaria mercenaria* (Olafsen et al., 1992).

Bivalves have an open circulatory system whereby the hemolymph passes out of the open end of arteries and bathes the organs before returning to the heart by way of the sinuses. Hemocytes are critical components of the immune system which play major roles in defense; they are also involved in nutritional and excretion processes. Brown et al. (1977) described gross disease manifestations due to hemic neoplasia in *Mya arenaria.* These included emaciation, watery tissues and mantle recession, suggesting a nutritional problem resulting from the burden of the neoplastic cells or a reduction in energy assimilation.

The bivalve immune system employs a large number of circulating molecules capable of cellular recognition and cytotoxic reactions (Pruzzo et al., 2005). The working combination of both cellular (hemocytes) and humoral components operate in a co-ordinated manner to provide protection from invading microorganisms (Pruzzo et al., 2005). More important, however, is the ability of these animals to adapt and conform to multiple environments and pathogens. These animals are osmoconformers as well as pathoconformers, enabling them to adapt and survive under variable conditions and in the persistence of variable levels of foreign intruders (Oliver and Fisher, 1999). For example, these animals are used as sentinels in pollution studies and are known to filter high levels of pollutants without negative consequences (Pipe, 1995). In some studies,

contaminant exposure can suppress defense activities, but in other studies, defense activities are enhanced (Oliver and Fisher, 1999).

Cellular defense has been shown to be present in the hemolymph as well as the extrapallial fluids, which is the fluid in contact between the mantle edge and the periostracal lamina (Allam et al., 2000). Hemocyte variation occurs in their ambient environment, which causes variability in hemocyte defense activities. Hemocytes also release lysosomal enzymes, opsonins in the form of lectins or agglutinins, anti-microbial peptides and reactive oxygen intermediates (Pruzzo et al., 2005). It is important to note that not all bivalve species are likely equipped with the same defense mechanisms. Due to incomplete knowledge on hemocytes, it is not known how defense functions are altered during the course of diseases. For example, the only functional defense studies on clams with hemic neoplasia have focused on adhesion and phagocytosis, which is altered in neoplastic hemocytes (Beckmann et al., 1992 and Moore et al., 1992). Reno et al. (1994) suggested that soft-shell clams experiencing hemic neoplasia are able to maintain phagocytosis to a certain degree. However, once the neoplastic cells reach 70-90% of hemolymph cellularity, phagocytosis is not possible. Further immune and physiological studies are needed to allow a better understanding of the implications hemic neoplasia has on the species. There has been speculation that hemic neoplasia causes immune compromise (Barber, 2004) but this has not been proven.

### *1.8.2 Cellular and humoral defense mechanisms of bivalve molluscs*

Phagocytosis is often described as the main defense mechanism in bivalve molluscs. It is a complex defense system required for neutralizing and eliminating foreign material, including organic and inorganic particles. Phagocytosis is the most studied aspect of cellular immunity in molluscs and has been found in all bivalve species (Chu, 2000). The percentage of hemocytes involved in phagocytosis can reach up to 90-100% in some bivalves including oysters, *C. virginica* and *C. gigas* (Yakovleva et al., 2001). Numerous studies have demonstrated that both agranulocytes and granulocytes can be phagocytic, but it is the latter are more active (Chu, 2000). The majority of bivalve species have been found to have approximately 70% granulocytes and 30% agranulocytes in the hemolymph (Hine, 1999; Wooton et al., 2003). The exception are clam species, *Tapes philippinarum* (Cima et al., 2000), *Meretrix lusoria* (Chang et al., 2005), and *Chamelea gallina* (Pampanin et al., 2002) in which agranular cells dominate. Phagocytosis is comprised of a series of events that include recognition of non-self, attachment, ingestion and intracellular killing (Chu, 2000). Phagocytosis may occur as soon as the host recognizes non-self or it could be a delayed process that occurs when energy is available. During phagocytosis there are two main mechanisms seen. They are the release of lysosomal enzymes and other lysins and/or the respiratory burst that involves the release of reactive oxygen metabolites such as superoxide anion and hydrogen peroxide (Carballal et al., 1997; Wooton et al., 2003).

*1.8.2.1 Recognition and attachment*

Hemocytes must be able to recognize and bind foreign objects in order to engulf them. Recognition of non-self by means of receptors mediated with the help of lectins are found in the plasma and on hemocyte membranes (Cheng, 1984; Roch, 1999; Yakovleva et al., 2001; Chu, 2000; Ordas et al., 2000). These allow direct attachment to the foreign object.

Lectins are specialized glycoproteins bearing multivalent binding carbohydrate motifs (Chu, 2000; Baier-Anderson and Anderson, 2000). The binding sites include one for non-self particles and one for hemocytes (Cheng, 1984 and 1996; Chu, 2000). Small pathogens are agglutinated or opsonized by lectins for enhanced clearance by circulati ng hemocytes or they can be lysed directly by enzymes in the hemolymph (Ordas et al., 2000; Pruzzo et al., 2005). Membrane and serum lectins have been described in *Mytilus edulis*, and *Crassostrea gigas, C. virginica* hemocytes (Cheng, 1984; Pipe, 1997) and in the serum of *Mercenaria mercenaria* (Olafsen et al., 1992).

*1.8.2.2 Ingestion*

Binding to a particle by a hemocyte initiates the process of phagocytosis. Hemocytes extend their pseudopods and wrap them around the particle to be engulfed. The particle is then contained within a phagosome. This phagosome than fuses with lysosomal granules forming a phagolysosome in which destruction of the particle occurs via the release of lysosomal enzymes and reactive oxygen intermediates (Chu, 2000). Lysosomal enzymes have been found in many bivalve species, including the clams *Mya*

*arenaria*, *Mercenaria mercenaria,* the oysters, *Crassostrea gigas, C. virginica and Ostrea edulis* and the mussels, *Mytilus edulis* and *M. galloprovincialis* (Lopez et al., 1997b). Pathogen engulfment by the cell requires active polymerization and remodeling of the actin cytoskeleton (Baier-Anderson and Anderson, 2000), which is apparently altered in neoplastic cells (Beckman et al., 1992).

*1.8.2.3 Intracellular killing*

Once the digestible material is degraded within the phagolysosome, the indigestible material is stored within cells or removed by diapedesis (Sinderman, 1990). The release of reactive oxygen metabolites through a process termed the respiratory/oxidative burst is induced by phagocytosis and/or membrane stimulation (Baier-Anderson and Anderson, 2000). This occurs through the production of oxygen radicals through the action of NADPH oxidase bound to cell membranes (Tafalla et al., 2003). It is characterized by activation of NADPH-oxidase, resulting in an increase in oxygen consumption and production of hydroxyl radicals, singlet oxygen and hydrogen peroxide (Roch, 1999; Chu, 2000; Mitta and Roch, 2000). These compounds are short-lived and can be directly toxic to both pathogens and cells (Mitta and Roch, 2000).

Encapsulation is another defense response. This is seen when an invasion from a parasite into the internal chamber of the shell causes the host to respond. The host will attempt to wall off the intruder to keep it from invading further. Encapsulation could be a delayed form of phagocytosis or a response to unsuccessful phagocytosis. Another important aspect of the molluscan humoral response is the production of antimicrobial

peptides (AMPs). Charlet et al. (1996) initially described the presence of AMPs and their role in innate immunity in the hemolymph of *Mytilus edulis*. To date, there have been four families of AMPs described: defensins, mytilins, myticins and mytimycin (Mitta et al., 2000). Anti-microbial activity is unique for a given class of pathogens and the killing properties rely on the disintegration of the microbial membrane (Charlet et al., 1996).

The study of bivalve immunity and how this immunity is compromised by hemic neoplasia requires a better understanding of the basic biology of these animals. Questions regarding neoplastic cell ontogeny, cell lineage and functional alterations caused by hemic neoplasia need to be addressed.

### 1.9 Neoplasia

In vertebrates, malignant neoplasia is recognized by proliferation of cells that constitute their parenchyma, involving supported stroma made up of connective tissue and blood vessels (Kumar et al., 2005). Morphologically, neoplastic cells range from well-differentiated to poorly differentiated (primitive looking, unspecialized cells) (Kumar et al., 2005). Differentiation refers to the extent to which neoplastic cells resemble similar normal cells both functionally and morphologically. Tumor characteristics include transformation of the target cell, rapid growth of these transformed cells, invasion and distant metastasis (Kumar et al., 2005). Transformation is a term describing the change from a non-neoplastic cell to a neoplastic cell (Cockerall and Cooper, 2005). Malignant tumors composed of undifferentiated cells are referred to as anaplastic. It is possible

that these undifferentiated cells have lost the instructions to fully mature. Features of anaplasia include pleomorphism (variation in size and shape), abnormal nuclear morphology (hyperchromasia, increase in N:C ratio, presence of single or multiple nucleoli), bi-nucleate cells, mitotic figures and loss of polarity (Kumar et al., 2005). These features are not always present at the same time and are not uniformly found in all malignant cells.

### 1.9.1 Molecular features of malignant neoplasia in vertebrates

Malignant neoplasia involves a process of several steps at the phenotypic and genotypic level. Due to multiple mutations in the DNA of these cells, malignant cells exhibit rapid unregulated growth. Multiple alterations involve activation of several oncogenes and loss of tumor suppressor genes (Kumar et al., 2005). Inactivation of tumor suppressor genes such as Rb and p53 in humans enable neoplastic cells to avoid responding to inhibitory signals, to evade apotosis, and to continue proliferating (Kumar et al., 2005). Unrestricted proliferative capacity is also associated with the maintenance of telomere length by the enzyme telomerase (Kumar et al., 2005). Neoplastic cells are capable of invading tissue and metastazing due to the unique changes that occur on the neoplastic cell membrane. Malignant neoplasia is an aggressive life threatening condition.

### 1.9.2 Other causes of disordered growth

Neoplasia is not the only disorder that causes cellular alterations and disordered growth. Other disorders share some of the morphological criteria of neoplasia but are non-life threatening and the cells in question lack the ability to invade tissue. However, some of

the morphological and behavioral characteristics of these disorders make them difficult to distinguish from neoplasia. Dysplasia (disordered growth) is a proliferative response accompanied by changes in cellular morphology and behavior. The cells display cellular atypia characterized by pleomorphism and hyperchromicity (Cockerall and Cooper, 2005). Loss in uniformity and architectural orientation is also common (Kumar et al., 2005). Morphologically distinguishing between severe dysplasia and neoplasia can be difficult. Another condition with some overlapping features with neoplasia is hyperplasia. It involves an increase in cells in an organ or tissue causing the tissue or organ to appear larger (Kumar et al., 2005). Hyperplasia occurs in cells capable of mitotic division and can be brought on by physiological (hormonal or compensatory) or pathological (hormonal or growth factor stimulus) responses (Kumar et al., 2005). Unlike neoplasia, this proliferation is controlled and regression occurs once the stimulus is removed. A condition seen frequently with hyperplasia is hypertrophy. Hypertrophy refers to an increase in the size of cells causing the organ to increase in size (Cockerall and Cooper, 2005). Cellular alteration is also caused by metaplasia. Metaplasia is an adaptive response whereby one adult cell type is replaced by another adult cell type (Kumar et al., 2005). During metaplasia the cell phenotype remains the same. Stem cells are re-programmed to differentiate along a new pathway with the help of signals produced by cytokines, growth factors and extracellular components (Kumar et al., 2005). In summary, dysplasia, hyperplasia, hypertrophy and metaplasia describe non-neoplastic morphological changes in cells. The ability to distinguish these conditions from neoplasia can be challenging.

### 1.9.3 Difficulties diagnosing hemic neoplasia in soft-shell clams

Presumed neoplastic disorders have been documented in bivalves for the last 30 years but many questions remain. It is possible that some of the neoplastic disorders documented in bivalves over the years are not true neoplasia, but are instead non-neoplastic changes such as hyperplasia. The diagnosis of neoplasia in bivalves is extremely challenging because of the lack of information regarding hematopoiesis, cell classification and ontogeny. Investigators often rely on mammalian characteristics and terminology for describing neoplasia. This often leads to discrepancies and lack of consensus in molluscan pathology.

Cells with abnormal quantities of DNA are termed aneuploid. In mammals, aneuploidy is involved in tumor formation because tumor cells are associated with different karyotypic changes and altered ploidy is often an indicator of malignancy (Bignold, 2007). Applying ploidy indicators as a sign of neoplasia in bivalves is challenging as aneuploidy can arise in situations other than neoplasia. Studies have shown that bivalves are naturally diploid (Bouilly et al., 2006). Exposure to chemicals such as diuron and atrazine (Bouilly et al., 2006) and elevated temperature (Yang and Gho, 2006) can lead to altered ploidy in bivalves. Further studies are required to determine the influence that exogenous factors have on ploidy in bivalves. Pentaploid and tetraploid cell populations have been observed in the blue mussel with disseminated neoplasia (Elston et al., 1990; Moore et al., 1991) while hypodiploid, hyperdiploid, triploid and pentaploid populations have been observed in the common cockle with this condition (Da Silva et al., 2005). Hemic neoplasia in the soft-shell clam is characterized

by a tetraploid phenotype. However, tetraploid cells are also found in healthy clams (Reno et al., 1994), making it difficult to distinguish between healthy clams and those affected with hemic neoplasia.

Diagnostic ambiguity also exists due to the lack of information on the normal hemocytes in *Mya arenaria*. Investigators studying hemic neoplasia have not reached consensus as to the morphology of normal hemocytes or to the variability of neoplastic cells. In fact, criteria are lacking regarding cellular disorders in general. The misapplication of mammalian terminology such as invasion and metastasis is misleading as these animals have an open circulatory system. Unlike the circulatory system of mammals, whereby organs and tissues are vascularized, an open circulatory system surrounds every organ in bivalves (Elston, 1990). Therefore, assessing the possible invasion of neoplastic cells into various tissues and organs can be difficult. The use of terminology such as metastasis should especially be avoided, as it is impossible to follow neoplastic cell movement from a primary site to a secondary site in bivalves. Inconsistencies in terminology and in interpretation of cellular disorders in bivalves over the years may have led an extremely confusing situation.

It is obvious that there have been numerous difficulties in trying to define hemic neoplasia in bivalves. Various techniques have been utilized in this attempt, including the use of monoclonal antibodies, assessment of phagocytosis and ploidy and disease intensity. However, no standardized criteria for definitive diagnosis of hemic neoplasia in bivalves yet exists.

Research on hemic neoplasia in the soft-shell clam is expected to contribute to basic science as well as to the development of an economically significant industry in Atlantic Canada. Given the difficulties in diagnosing hemic neoplasia and distinguishing it from other proliferative disorders, several objectives were established for the present study. A thorough morphological description and classification of normal soft-shell clam hemocytes is needed before neoplastic hemocytes can be adequately described and classified. The use of cytology, electron microscopy and histology can be used to aid in this endeavour. Flow cytometry also offers the potential for a quantitative diagnosis of hemic neoplasia once a protocol is established. The specific aims of this study were therefore to:

1. Apply a flow cytometry protocol for evaluating hemic neoplasia.
2. Establish disease classification through the development of a flow cytometer case definition for hemic neoplasia for individual clams and populations.
3. Describe and classify normal hemocytes and neoplastic hemocytes by cytology, electron microscopy and histology.

# Chapter 2

## Field study of hemic neoplasia in six soft-shell clam populations with flow cytometry

### 2.1 Introduction

Hemic neoplasia has been found in several soft-shell clam populations in Prince Edward Island (PEI) with a high disease prevalence and intensity (McGladdery et al., 2001). In the Gulf of St. Lawrence, this disease has also been found in clam populations with low prevalence and disease intensity (McGladdery et al., 2001). The etiology of hemic neoplasia is believed to be multi-factorial for a given time and place. Studies have attempted to associate the occurrence of hemic neoplasia in soft-shell clam populations to anthropic activities. Although studies exist that support a pollution etiology (Yevich and Barszcz et al., 1977; Harshbarger et al., 1979; Strandberg et al., 1998), there are studies that have failed to find an association (Appeldoorn et al., 1986; McGladdery et al., 2001). Environmental influences, including salinity, temperature and sediment type, can be considered possible risk factors. Sudden changes in these variables have been shown to have negative impacts on bivalves (Leavitt et al., 1990; Pipe, 1995).

The diagnosis of hemic neoplasia is potentially achieved with cytology, histology and flow cytometry. Cytology is a non-lethal technique that provides a visual examination of the hemocytes. It requires that the observer is able to distinguish between normal and neoplastic hemocytes. The recognition of neoplastic cells is based on their enlarged size, high nucleus to cytoplasm ratio and hyperchromasia. Disease progression can be followed temporally with disease intensity determined by the percentage of abnormal

cells of the total hemocyte percentage. Histology allows evaluation of the severity of hemic neoplasia by determining the extent and location of neoplastic cells in the tissues. Histology, however, is a lethal test and is time demanding. This limits its use in some epidemiological studies which require rigorous sampling of large numbers of animals.

Both cytology and histology provide a framework of reference for comparing normal and abnormal conditions. With these techniques, observer objectivity and methodological variations occur. Difficulties in interpretation can lead to inconsistent results. A number of interpretative scales have been published for the presence and intensity of hemic neoplasia using cytology (Farley, 1969 a and b; Leavitt et al., 1990; Brousseau and Baglivo, 1991; White et al., 1993; McGladdery et al., 2001; McGladdery and Davidson, 2003) and histology (Mix, 1983; Farley et al., 1986).

Flow cytometry may be an effective tool for diagnosing hemic neoplasia. Unlike previous methods, flow cytometry allows for a high throughput objective examination of the DNA content of thousands of cells per second from a small volume of hemolymph. The ability to screen large numbers of cells greatly exceeds the capacity of traditional methods and offers a reliable, non-lethal examination of each animal. However, flow cytometry requires specialized training for protocol development and technical support.

Flow cytometry has potential for investigating bivalve hemocyte populations, activities and functions (Ashton-Alcox and Ford 2001; Xue et al., 2001; Delaporte et al., 2003;

Hegaret et al., 2003). Several studies have used flow cytometry for evaluating the DNA content of normal and neoplastic cells in a number of mollusc species (Elston et al., 1990; Reno et al., 1994; Da Silva et al., 2005). The protocol most commonly used to detect hemic neoplasia is based on DNA quantification with the use of a fluorescent dye as propidium iodide (Reno et al., 1994; Da Silva et al., 2005; Smorlarz et al., 2005). The blue mussel, *Mytilus edulis*, displays a tetraploid and pentaploid DNA content (Barber, 2004) when affected by hemic neoplasia. Da Silva et al. (2005) established that the common cockle, *Cerastoderma edule,* displays various ploidy levels when experiencing hemic neoplasia. These include hypodiploid, hyperdiploid, triploid, tetraploid and pentaploid variants.

The soft-shell clam, *Mya arenaria*, has been reported to have a tetraploid (4N) phenotype in situations of hemic neoplasia (Reno et al., 1994). Although a tetraploid hemocyte phenotype has been reported in soft-shell clams with hemic neoplasia, this is not the only situation which can result in an abnormal phenotype. Normal hemocytes have the ability to undergo mitosis while in circulation, and may extend their time in the S phase of the cell cycle preparing for division. These cells have replicated their DNA but have not yet divided; they are thus tetraploid (4N). Reno et al suggests that 4-6% of hemocytes in normal clams are in the S phase with a resultant 4N phenotype (Reno et al., 1994). It is not known if non-neoplastic proliferative situations such as hyperplasia increase these percentages. It is also not known at what higher percentage a diagnosis of hemic neoplasia can be supported. This situation is further complicated by the lack of understanding of hematopoiesis and stages of hemocyte maturation in soft-shell clams.

Without percentage tetraploid thresholds defining hyperplasia and neoplasia, the results of ploidy evaluation via flow cytometry would be difficult to interpret. The weakness here is not necessarily a result of the tool but a result of the lack of an established threshold separating hemic neoplasia negative from positive clams on an individual and population level. Flow cytometric data is particularly difficult to interpret when a large diploid peak in the $G_0/G_1$ phase of the cell cycle is seen along with a slight increase in cells. The presence of low levels of tetraploid cells may be due to early neoplasia, hyperplasia or normal variation. These ambiguous results make it difficult to diagnose early hemic neoplasia in the soft-shell clam. Advanced cases may be more obvious to identify if high percentages of tetraploid cells are seen in the S phase. The main objective of this study was therefore to develop a case definition using tetraploid thresholds for hemic neoplasia at the individual and population level. Although there will be false positives and negatives, these thresholds will provide case definitions to assign clams to positive and negative groups.

A study evaluating potential hemic neoplasia was conducted on six populations of soft-shell clams from Atlantic Canada using flow cytometry and cytology. Individual clams displaying <1% neoplastic cells as determined by cytology are considered to be negative to early positive for hemic neoplasia (Farley, 1969 a and b; Brousseau and Baglivo, 1991; McGladdery et al., 2001). For the present study, this literature case definition of <1% neoplastic cells was used to potentially confirm the flow cytometry results, with the purpose of establishing a case definition for hemic neoplasia on an

individual and population level. Although flow cytometry provides the ability to assess large numbers of clams in a non-lethal fashion, it was important to use cytology as a visual reference to potentially confirm the flow cytometry results. Including a traditional method like cytology offers another layer of insight into the overall situation and improves the level of diagnostic certainty. Although we considered cytology as a potential confirmatory tool, it was necessary to evaluate the diagnosis by the level of agreement between both techniques.

## 2.2 Materials and Methods

### 2.2.1 Animals

Six soft-shell clam populations were collected at low tide and brought to the aquatic facility at the Atlantic Veterinary College in Charlottetown, PEI. Sixty soft-shell clams were collected from each site. These clams ranged from 50-60 mm in shell length. Each clam population was held in separate re-circulation tanks for 3-4 weeks at a temperature of 18° C and fed an algae paste three times per week. Sites were chosen on the basis of geographic variation and anecdotal reports of the levels of hemic neoplasia. Sites chosen for this study are shown in Figure 1.

### 2.2.2 Hemic neoplasia diagnosis

#### 2.2.2.1 Hemolymph preparation

A sample of approximately 1 ml of hemolymph was withdrawn from the anterior abductor muscle of each clam using a 21-gauge 5/8" needle fitted with a 3 ml syringe. Each sample was utilized for both flow cytometry (0.5 ml) and cytology (approximately

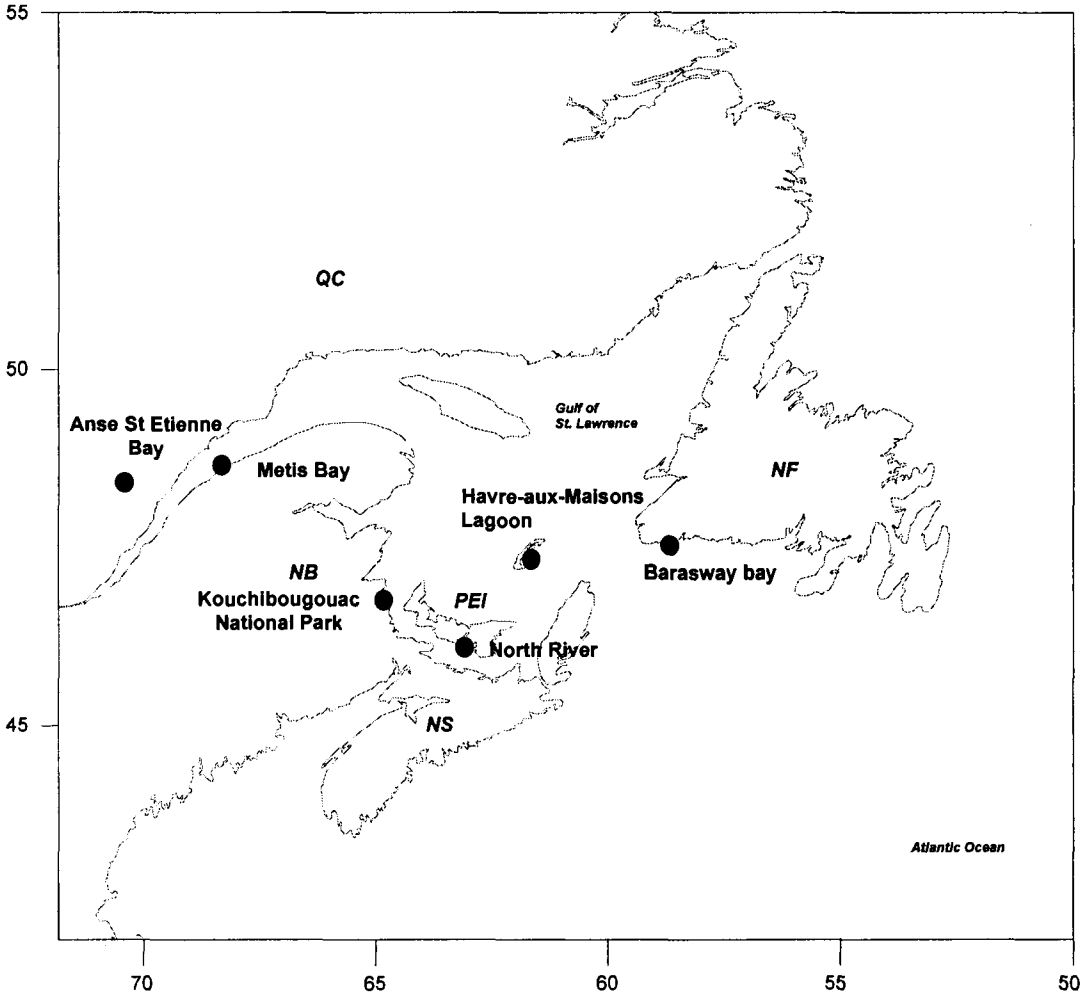


**Figure 1**: Map displaying 6 sampling locations in Atlantic Canada.

Anse St-Etienne, Saguenay Bay, QC (69W11°, 48N35°)

Metis Bay, QC (68W11°, 48N35°)

Havre- aux-Maisons lagoon, Magdalen Island, QC (61W47°, 47N26°)

Kouchibougouac National Park, NB (64W81°, 46N67°)

North River, PEI (63W08°, 46N14°)

Barasway Bay, Burgeo, NL (57W38°, 47N36°)

150 µl). The remaining volume from each sample was then transferred to 15 ml centrifuge tubes, vortexed to mix the sample and held on ice.

2.2.2.2 Flow cytometry

A sample of 0.5 ml of hemolymph were added to 2 ml of 95% cold ethanol and held at - 20°C for 24-72 hours. Samples were prepared according to Da Silva et al. (2005). Samples were thawed for 15 minutes at room temperature, then centrifuged at 400 g for 10 minutes at 5°C to pellet the cells. The sample was decanted to remove ethanol, then the pellet of cells were re- suspended in 380 µl of phosphate buffered saline (PBS) (91 ml of Solution A: $KH_2P04$, 0.01M (1.36 g in 1 L of deionized water; 409 ml of Solution B: $Na_2HP04$, 0.01M ph 7.4) at room temperature. Hydration of the cell suspension occurred for 30 minutes at room temperature, followed by two washes in PBS (0.01M) and centrifugation at 400 g for 10 minutes. The sample was then transferred to flow tubes (BD Falcon) filtering through 80 µm nylon mesh to eliminate debris. Cells were treated with 1.9 µl of DNase- free RNase A (Sigma, R4875, 50µg/ml) to eliminate interfering cellular RNA, and stained with 19 µl of propidium iodide (PI) (Sigma, P4170, 50 µg/ml). Propidium iodide is a fluorescent DNA/RNA specific dye that permeates through the membrane of dead cells and stains nucleic acid. Samples were incubated at room temperature for 30 minutes in the dark to allow optimal staining. A specific FL2 detector (550-600 nm) of a FACSCalibur (Becton BioScience) flow cytometer was used to measure fluorescence of stained cells. A single electronic pulse of PI provided DNA quantification of each cell. Each pulse is discriminated by its area, height and width. Using this flow cytometer, replication status of cells can be achieved

by fluorescence labeling of the nuclei of these cells in suspension. One copy of DNA is found in quiescent and G1 (resting) cells and these eukaryotic diploid (2N) cells have 1X fluorescence intensity. Cells in S phase are synthesizing DNA. These cells have fluorescence values between the 1X and 2X populations. Two copies of DNA are found in cells in the $G_2$/M (pre-mitotic or mitotic) phase of the cell cycle; these cells are tetraploid and have 2X intensity. Light scatter analysis involved two parameters: forward and side scatter. Forward angle scatter (FSC) and side angle scatter (SSC) gating on the single cell region (R1) FL2 (width) versus FL2 (area) dot-plots provided information on cell size and complexity. Representation of the width and area of the PI fluorescence pulse and distinction of diploid (2N) cells and tetraploid (4N) cells separated from doublets was shown on cytogram graphs. Once gated on the R1, the PI fluorescence of the single cell population was plotted on a FL-2 area histogram, where a specific marker was situated to have the number and percentage of S phase cells in this region. Disease intensity of hemic neoplasia for each population was based on the percentage of S phase - 4N cells found in each individual clam. Samples were run at low flow rate (15 $\mu l\ min^{-1}$). For quality control, samples that had less than 700 cell events measured at low speed were eliminated from the study.

Flow data was analyzed with flow cytometric software (FACS Express). Based on cytogram contours and appearance representing the width and area of the PI fluorescence signal, the pattern for negative clams with normal diploid parameters consisted of one plot of single cells with 2N DNA content in the R1 region (Figure 2a: Appendix A). For the histogram analysis, one major peak (MI) was observed that

represented the intensity of PI fluorescence (Figure 2b: Appendix A). Clams potentially positive for hemic neoplasia were represented by one plot of single diploid and one plot of tetraploid cells observed in R1 and M2 on the cytogram and histogram (Figure 2c and 2d: Appendix A).

2.2.2.3 Cytology

Three drops (approximately 150 µl) of hemolymph were added to 2 ml PBS (NaCl, 29.45 g, $Na_2HP0_4$ anhydrous, 1.48 g, $KH_2P0_4$, 0.43 g, pH-7.6) while vortexing. The contents of each tube were poured into to 25 mm histoprep embedded rings attached to poly L-lysine coated slides and incubated for one hour in a moist chamber at room temperature. The slides were then removed from the histoprep embedded rings and gently blotted to remove excess solution. Slides were then fixed for 2 minutes in methanol at room temperature and stained with Phosphate buffered Eosin solution (1 minute) and Phosphate buffered Thiazine solution (1 minute) (Harleco, Fisher VWR). Slides were mounted with a histological mounting medium (Permmount, Fisher Scientific, New Jersey) and examined. Up to 600 - 1100 cells per slide were counted over five fields with a manual counter to determine the percentage of neoplastic cells. Slides that had too few cells, clumping or poor staining were considered of poor quality and were discarded. Normal and cells deemed to be neoplastic were subjectively distinguished based on morphological appearance. Negative hemocytes typically had a low nucleus to cytoplasm (N: C) ratio and pseudopodia. Clams deemed to be neoplastic were larger, approximately 1.5 - 2x the diameter of negative hemocytes, and had high N: C ratios. Compared to negative hemocytes, the nuclei of these presumed neoplastic

cells were deeply basophilic. Some of these presumed neoplastic cells also lacked pseudopodia. These cells were deemed to be neoplastic for the purpose of this study and are referred to as neoplastic throughout the rest of the chapter. It is important to note that although these cells were deemed to be neoplastic, they may not necessarily be true neoplastic cells. A scale was developed based on the percentage of presumed neoplastic cells: Stage 0: few neoplastic cells 0 - 0.99% (negative), Stage 1: 1 – 9.9% (low level) Stage 2: 10 - 49% (light - moderate) Stage 3: 50+ (heavy).

2.2.2.4 Determination of case definitions for individual clams and populations

To determine a case definition of hemic neoplasia for flow cytometry, the results obtained with the flow cytometer from each population were potentially confirmed with cytology. The case definition for a negative clam using cytology was <1% neoplastic cells of the total hemocytes. This case definition was used to obtain the level of agreement between cytology and flow cytometry. Reno et al. (1994) using flow cytometry reported that approximately 4-6% of hemocytes in normal clams are in the S phase and are expressing a 4N phenotype. Therefore, to determine the highest level of agreement between both techniques for individual clams, the flow cytometry and cytology results were compared using 4%, 5% and 6% 4N thresholds. The test results were evaluated with the use of 2x2 tables. Cytology was considered the potential confirmatory tool and flow cytometry the test status. The 4N percentage with the highest level of agreement was planned to be used as the cut-off threshold for the study.

Flow cytometric studies in our laboratory, utilizing cell cycle software, suggest that clams with >20% 4N cells may truly have hemic neoplasia (Delaporte et al., publication in preparation). Clams with <20% 4N cells may have non – neoplastic processes such as hyperplasia; this is speculative at this point. To evaluate disease intensity between populations, an additional threshold was selected requiring >25% of cells from at least one clam to be 4N to designate the population as having hemic neoplasia. This threshold was arbitarily chosen to be 5% higher than the >20 % 4N identified in our laboratory to hopefully identify hemic neoplasia more reliably. Due to the level of difficulty of distinguishing true neoplasia from other cellular disorders based on ploidy and morphology, clams with a high ploidy percentage and abnormal hemocytes on cytologic evaluation were presumed to have neoplasia using these cut-offs. These clams will be referred to as positive throughout the rest of this chapter.

2.2.2.5 Statistical Analysis

Statistical analysis was performed with Minitab 14 (Minitab Inc., Pennsylvania). One-way analysis of variation (ANOVA) on log-transformed data was used to test for significance between sample populations based on the percentage of 4N cells of individuals from each population. Significance was set at $p < 0.05$. Pairwise comparisons were made between each site. Normal distribution and homogeneity of variances were previously tested on the data.

The best agreement and combination of sensitivity and specificity between cytology and flow cytometry was achieved at the 5% threshold (details to be presented in section

2.3.1). This 5% 4N threshold was thus used in various statistical analyses to compare differences between the sites on an individual and population level. The 25% cut off was used to evaluate disease intensity on a population level. A population was considered positive for hemic neoplasia if more than one clam displayed a disease intensity of >25% 4N cells. A strategic sampling method was not common among all sites, therefore, the disease prevalence may not be completely representative within all sites. However, the apparent differences in disease prevalence (the total number of clams positive for hemic neoplasia in a population, divided by the number of clams in that population) was determined with a Fishers exact test for proportions available for 2x2 tables with Bonferroni adjustment for 15 different comparisons (0.05/15= 0.003) (p= 0.003). The mean disease intensity (the percentage of tetraploid cells of total hemocyte count) of each site was compared using a one-way ANOVA (p=0.000) on data >5% 4N for each site. The various hemic neoplasia thresholds are summarized in Table 1.

**2.3 Results**

2.3.1 Population Study

One hundred and twelve clams were discarded from this study leaving 248 clams. These samples were discarded due to poor quality staining, too few cells and cell clumping. Samples with <700 cells/ sample were discarded from flow cytometry analysis. Approximately half of the samples discarded were due to poor staining and cell clumping.

**Table 1**: Hemic neoplasia thresholds utilized in the study.

| Method and assessment goal | Deemed negative for hemic neoplasia | Deemed positive for hemic neoplasia |
|---|---|---|
| Cytology | An individual clam has <1% cells of total hemocytes visually classified as neoplastic | An individual clam has ≥1% of total hemocytes visually classified as neoplastic |
| Agreement between cytology and flow cytometry | An individual clam has <5% 4N cells on flow cytometry and <1% of the total hemocytes visually classified as neoplastic by cytology | An individual clam has >5% 4N cells on flow cytometry and >1% of the total hemocytes visually classified as neoplastic by cytology |
| Flow cytometry for evaluating populations | All clams have ≤ 25% 4N to designate the population as negative | At least one clam has ≥ 25% 4N to designate the population as positive |

To determine agreement between cytology and flow cytometry, the results from all 6 sites were combined. A 88% agreement between flow cytometry and cytology was determined with the 4% case definition. With this case definition, there was a sensitivity of 83% and specificity of 89%. A 93% agreement between flow cytometry and cytology was determined with the 5% case definition. With this case definition, a sensitivity of 80% and specificity of 98% was found. A 90% agreement between flow cytometry and cytology was determined with the 6% case definition. With this case definition, a sensitivity of 66% and specificity of 99% was found (Refer to Table 2: Appendix B for complete summary).

The Saguenay Bay site was the only site in which all clams had <5% 4N cells. These results were confirmed with cytology as being negative for the presence of presumed neoplastic cells. Based on the agreement and combination of sensitivity and specificity and the results from Saguenay Bay, it was decided that for this study a case definition of 5% 4N cells, on an individual level, was the most appropriate. Therefore, utilizing the flow cytometer, a clam with >5% 4N cells is considered positive for hemic neoplasia and will be referred to as positive throughout the rest of this chapter.

2.3.1.1 Case definition at a population level

The proportion of individuals above 5% 4N cells, disease prevalence and intensity for each population was determined and statistically evaluated. Saguenay Bay had a statistically lower disease prevalence than all other sites ($p = <0.003$) (Figure 3, Table 3). There was no significant difference in disease prevalence found between the

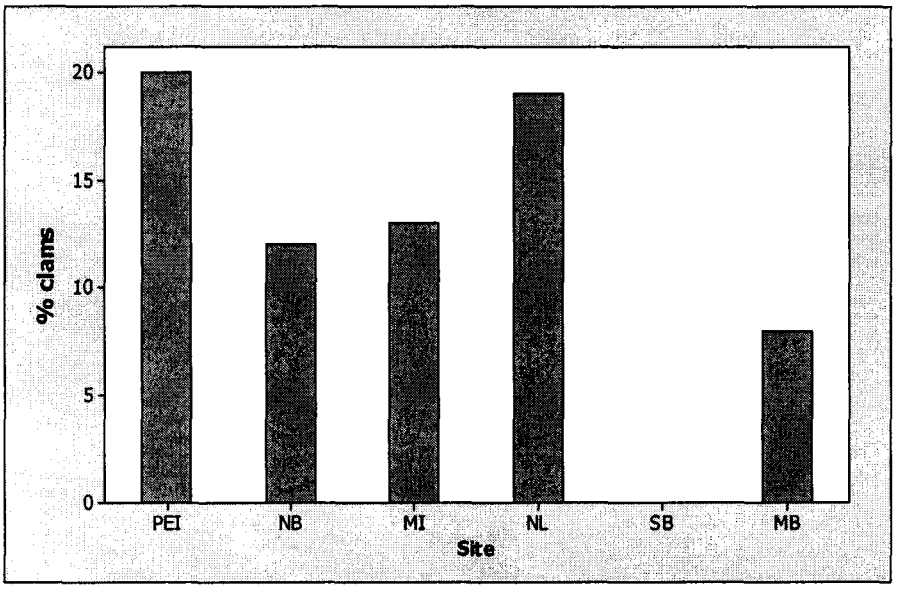


**Figure 3**: Graphical representation of the proportion of individual clams with > 5% 4N cells from each population. According to the case definition for individual clams, the Saguenay Bay site is statistically different from all other sites (n= 42 clams; Fisher's exact test; p= <0.003). There was no significant difference between the remaining sites (p=>0.003). PEI= Prince Edward Island, North River site; NB= New Brunswick, Kouchibouguac site; MI= Magdalen Islands, Havre-aux-Maison site; NL= Newfoundland, Barasway Bay site; SB, Que= Saguenay Bay, Quebec, Anse St-Etienne site; MB= Metis Bay, Quebec.

**Table 3**: Population summary of hemic neoplasia diagnosed by flow cytometry at 6 sites

| Site | N | Mean % | Median % | Stdv. % | Disease Prevalence >5% 4N | Mean Disease Intensity | Percentage > 25% 4N |
|---|---|---|---|---|---|---|---|
| Saguenay Bay, QC | 42 | 1.445 | 1.235 | 0.891 | 0/42 = 0% [A] | N/A | 0 |
| Kouchibouguac, NB | 34 | 3.988 | 3.910 | 1.874 | 12/34 = 35% | Mean 6.09 95% (4.45, 7.73) | 0 |
| Metis Bay, QC | 47 | 3.157 | 2.810 | 2.800 | 8/47= 17% | Mean 7.81 95% (0.93, 14.69) | 0 |
| Havre-aux-Maisons, QC | 45 | 3.852 | 2.660 | 3.221 | 13/45= 29% | Mean 7.93 95% (2.07, 13.79) | 0 |
| Burgeo, NL | 43 | 4.923 | 3.840 | 3.546 | 19/43= 44% | Mean 8.14 95% (2.28, 14) | 0 |
| North River, PEI | 57 | 12.45 | 3.62 | 21.33 | 20/57= 35% | Mean 31.02 [B] 95% (-24.82, 86.86) | 16 [C] |

**Note**: Sites with letters A, B and C were statistically different at 5% than all other sites.

remaining sites (p= >0.003). From an individual perspective, according to the case definition for this particular study, the clams from Saguenay Bay sample were the only "true" negative sample.

Based on mean disease intensity, the North River site was determined to be statistically different from all other sites (p= <0.005). There was no significant difference between the mean disease intensity of the remaining sites (p= >0.005) (Table 3). To further evaluate the difference between the disease intensity from a population perspective, populations were compared using the additional threshold of >25% 4N cells. The mean disease intensity of the North River site was determined to be statistically different at >25% 4N level (p=0.003) than all other sites. The disease intensity for the remaining sites was < 17% 4N cells. Statistical data for the population survey is summarized in Table 3. Cytological analysis of clam hemolymph from North River site resulted in as many as 78% neoplastic cells. Results from Metis Bay and Havre-Aux-Maisons clams showed <19% neoplastic cells. Clams from Kouchibouguac and Burgeo, had < 8% neoplastic cells. Analysis with cytology led to a slight over-estimation of neoplastic cells in each sample compared to the flow cytometer.

**2.4 Discussion**

The individual case definition using flow cytometry for a clam positive for hemic neoplasia in this study is having greater than 5% of the hemocytes as tetraploids. This was guided by a Reno et al. (1994) study that determined a normal clam can have approximately 4 - 6% of hemocytes cells expressing a tetraploid phenotype. Reno et al.

(1994) combined a Modfit cell cycle analysis with the flow cytometer settings in their study. These authors determined the proportions of cells in the $Go/G_1$ and $G_2$ /M and suggested that about 4% 4N cells are in the S phase and 2% 4N cells in $G_2$ /M for a total of 6% 4N cells for healthy clams. We consider that during the cell cycle it is normal to find up to 5% 4N cells in the S phase or pre-mitotic phase.

To establish a population case definition for clams positive for hemic neoplasia, intensity of the disease in individual clams as well as prevalence in a population must be considered. Using the case definition defined in this study for individual clams, the Saguenay Bay site was the only sample with all clams diagnosed as hemic neoplasia negative. The North River site, with a prevalence of 35% and a mean disease intensity of 31% in positive clams, can safely be classified as a positive hemic neoplasia population. This population is an impacted site that is closed for aquaculture and recreational clam fishing because of fecal coliform contamination (McGladdery et al., 2001). A study by McGladdery and Davidson. (2003) concurs with the present study, reporting a 95% prevalence of hemic neoplasia and a high mortality in the North River clam population in July 1999.

The other populations studied were not possible to classify as disease positive or negative, as the prevalence of the disease was often high (17 - 44%) but the mean disease intensity was low (6.09 - 8.14%). These sites may be considered disease positive due to high disease prevalence or disease negative due to low intensity. Although prevalance is a useful parameter, the intensity of hemic neoplasia in clams

may offer more information on the status of the disease in clam populations. Further studies are required to determine the role that prevalence, intensity and their interaction contribute to population disease classification.

Prevalence and disease intensity can be affected by many variables such as temperature, sediment type, stocking density, food availability, anthropic activities and mortality. Therefore, it is important to recognize that this study provides a single snapshot view of the situation and although these populations may be considered positive, they are not necessarily expressing the disease in the form of mortality. Nevertheless, whenever there is an increased prevalence it is important to assess the status of these populations on a seasonal basis. This is important as hemic neoplasia is documented as having a seasonal pattern (Barber, 2004). Future studies with larger sampling sizes, more geographical sites, habitat comparisons and multiple sampling times throughout the year are necessary.

To determine the spatial and temporal distribution of hemic neoplasia in Prince Edward Island, an extensive field survey was conducted in 2000 (McGladdery et al., 2001). This study reported the results from data collected from 1997 - 2000 for 28 sites. Prevalence varied in sites during different sampling times. For example, clams from Baltic River showed prevalence ranging: 0 - 20% in June of 1997, 5% in June and July of 1998 and 80% in July of 1999 (McGladdery et al., 2001). Geographic location, season, environmental parameters, mortality rate and clam susceptibility may explain this

variation. Future studies focusing on these parameters and how they relate to prevalence is required.

With or without a case definition, defined for a disease in a population, misclassifications can occur. The case definition is arbitrary depending on the objective of the study. For example, a transmission study designed to strictly evaluate if the population is disease negative may have a case definition of requiring less than 1% of positive cells in all clams. Although this designates the population as negative, it may increase the number of truly negative populations that will be classified as positive (false positive). On the other hand, if the objective is to ensure that all negative clams are identified, the case definition may be a prevalence of 10% with at least 10% of the clam population having a disease intensity of 25%. Although this will identify all the negative populations, there will be a greater risk of classifying positive populations negative (false negative). As the case definition varies, the sensitivity and specificity has an inverse relationship (Fagen, 2001). As the sensitivity increases, the specificity decreases and vice versa, depending on where the cut-off point separating negative from positive is placed.

Measurement biases exist due to the lack of information on bivalve hematopoiesis, lack of information on the normal morphology of hemocytes in the soft-shell clam and our case definition. The case definition for hemic neoplasia is especially problematic because a slight increase in DNA content above the 5% 4N cut-off may reflect a non-neoplastic physiologic process rather than hemic neoplasia. Differentiating between

these potentially negative or positive clams has never been addressed. The majority of studies on hemic neoplasia utilize clams with a high neoplastic cell percentage. Faulty conclusions could also arise due to the lack of knowledge on S phase measurement using flow cytometry for the soft-shell clam. Furthermore, these ambiguous results make it difficult to gather information from an epidemiological perspective on the situation of hemic neoplasia in Atlantic Canada. Using a cut-off of 4N ploidy percentage here is potentially useful. The present study is the first study to offer a case definition for separating negative and positive clams. A weakness, however, is that the case definition does not provide information on disease progression. Development of other markers for hemic neoplasia should improve the understanding of this disease. In particular, antibodies specific to non-neoplastic and neoplastic cells could be used to follow disease progression of individual and/or populations. In addition, future studies with larger sampling sizes, more geographic sites and habitat comparisons may offer insight into the impact hemic neoplasia has on soft-shell clam populations in Atlantic Canada.

## Chapter 3

## Morphological study of hemocytes of the soft-shell clam, *Mya arenaria,* via light and electron microscopy and histology

### 3.1 Introduction

Literature on bivalve immunity and physiology is abundant, with several published reviews summarizing the information on the types and functions of hemocytes (Cheng, 1981 and 1996; Auffret, 1988; Ford and Tripp, 1996; Hine, 1999; Chu, 2000). These reviews emphasize the major roles hemocytes play in defense, nutrition and excretion. The majority of investigators agree that granulocytes and agranulocytes are the two main types of hemocytes in bivalves (Hine, 1999; Chu, 2000). However, many questions remain regarding hematopoiesis, cell lineages and sub-populations (Hine, 1999).

Bivalve hemocyte classification is often based on morphology, using light microscopy and transmission electron microscopy. Other studies have used lectin binding properties (Cheng, 1981; Pipe, 1997; Wooton et al., 2003; Pruzzo et al., 2005) and enzymatic characterization for classification (Cheng, 1981; Ordas et al., 2000; Cima et al., 2000; Pampanin et al., 2002; Wooton et al., 2003). The use of flow cytometry has also been helpful in bivalve cell studies. Unlike conventional microscopic techniques, flow cytometry allows rapid analyses of the morphological characteristics of hemocytes in high numbers of cells. Investigators have separated hemocyte populations using light scatter parameters such as forward scatter (FSC) and side scatter (SSC) related to cell size (FSC) and complexity (SSC), in the oysters, *Crassostrea virginica, C. gigas* and

*Ostrea edulis,* the cockle, *Cerastoderma edule* (Ford et al., 1994; Xue et al., 2001; Ashton-Alcox and Ford, 1998) and scallop, *Pecten maximus* (Auffret, 1988).

*Reliance on total hemocyte count*

The total hemocyte count may be used to monitor changes in response to stress and disease in both clinical and research settings (Battison et al., 2003). Divergent results have been reported for differential hemocyte counts in bivalves (Hine, 1999). Unlike vertebrates, bivalves conform to changes in their environment and thus, the total hemocyte count may change in response to season and environmental influences (Oliver et al. 2005; Cao et al., 2007). The validity of total and differential hemocyte counts will be addressed further in the discussion of this study.

Hemic neoplasia transforms hemocytes in the soft-shell clam. Hemic neoplasia is characterized by increased numbers of abnormal cells in the hemolymph and tissues (Brown et al., 1977; Cooper et al., 1982; Reinisch et al., 1983; Farley et al., 1986; Elston et al., 1992; Barber, 2004). Cells described as neoplastic are morphologically and functionally altered compared to normal hemocytes (Brown et al., 1977; Cooper et al., 1982; Reinisch et al., 1983; Farley et al., 1986; Elston et al., 1992; Barber, 2004). These cells are described as lacking the ability to carry out normal functions such as adhesion and phagocytosis (Beckmann et al., 1992). The evaluation of hemocyte morphology and how this morphology is altered during the course of hemic neoplasia is an important aspect in understanding the pathogenesis of this disease.

Investigators studying hemic neoplasia have described the morphology and behavior of neoplastic cells, while the biology of normal hemocytes is not yet fully understood. Few studies have investigated the types and respective functional roles of hemocytes in the soft-shell clam (Huffman and Trip, 1982; Seiler and Morse, 1988). Therefore, questions regarding the types of hemocytes, lineages, roles and ontogeny remain. The minimal information available regarding normal hemocytes made it difficult to distinguish between normal and neoplastic cells during this project. Before achieving the goal of identifying neoplastic hemocytes by morphology, it was first necessary to describe and classify the types of hemocytes in clams free from hemic neoplasia. Evaluation of these normal hemocytes provided descriptive information which was then useful for recognizing neoplastic cells. Thus, the development of specific criteria for recognizing non-neoplastic and neoplastic cells should provide knowledge on the types of hemocytes in the soft-shell clam and how these hemocytes are morphologically altered during hemic neoplasia.

## 3.2 Materials and Methods

### 3.2.1 Animals

Clam populations at two sites were sampled for this study: Havre-aux-Maisons lagoon, Magdalen Islands, QC and North River, PEI. The Magdalen Islands population was selected because it has historically had a low prevalence of hemic neoplasia (Myrand, personal communication). Healthy clams were therefore obtained from this site with confidence. The North River population has historically been shown to have a high intensity and prevalence of hemic neoplasia (McGladdery et al., 2001). Hemic neoplasia

positive clams at various levels of disease intensity were obtained with confidence from this site. Clams ranging from 50 – 60 mm in shell length were sampled from each population. Individual clams were assessed for hemic neoplasia with flow cytometry with hemic neoplasia recognized by tetraploidy. In chapter 2, the flow cytometer case definition for an individual clam deemed positive for hemic neoplasia was >5% 4N cells. However, this case definition can be arbitrary depending on the objective of the study. In the present study, to ensure clams were hemic neoplasia negative, only clams with <1% 4N cells as assessed by flow cytometry were selected. A total of 110 clams from both sites were utilized for the study. This sample number provided sufficient working material to evaluate the type of hemocytes and neoplastic cells in negative and positive clams. For the cell study, 40 clams were sampled from Havre-aux-Maisons and 40 clams from North River. Hemic neoplasia status was determined by flow cytometry. The hemocytes were analyzed by light and transmission electron microscopy. For histology, a second group of 10 clams from Havre-aux-Maisons and 20 clams from North River were sampled. These clams were analyzed by flow cytometry to determine hemic neoplasia status and prepared for histology.

*3.2.1.1 Havre-aux-Maisons lagoon, QC*

For the cell study, clams were collected at low tide at this site (61W47°, 47N26°) in late May 2006. These clams were held on ice for approximately 3-5 hours while shipped to the Atlantic Veterinary College, University of Prince Edward Island. Upon arrival, clams were held in a recirculation tank for up to one week at a temperature of 17-18°C and fed an algae paste before being assessed for hemic neoplasia. A second sampling

was carried out in September 2006. These clams were held in a recirculation tank for up to one week at a temperature of 17-18°C and fed an algae paste before being assessed for hemic neoplasia. Clams were assessed for hemic neoplasia using flow cytometry and cytology; they were then processed for histology (n=10).

*3.2.1.2 North River, PEI*

Clams were collected at low tide at this site (63W08°, 46N14°), between the months of June and early September 2006. Upon arrival, clams were held in a recirculation tank for up to a week at a temperature of 17-18°C and fed an algae paste before being assessed for hemic neoplasia. Positive clams ranged from 1-85% 4N cells (n=40) as determined by flow cytometry. In late September of 2006, 20 additional clams were collected and assessed for hemic neoplasia using flow cytometry; they were then processed for histology.

3.2.2 Hemic neoplasia status assessment

3.2.2.1 Hemolymph collection

A sample of 0.5 ml of hemolymph was withdrawn from the anterior adductor muscle of each clam using a 21-gauge 5/8" needle with a 3 ml syringe. This hemolymph sample was then transferred to 15 ml centrifuge tubes containing cold ethanol (volume: 1:5) and vortexed to mix the sample. Samples were held at - 20°C until further use.

3.2.2.2 Flow Cytometry

Samples were allowed to thaw for 15 minutes at room temperature and processed identically to that described in section 2.2.2.2.

3.2.3 Light Microscopy

3.2.3.1 Hemolymph collection

For the cell classification study, hemolymph from 40 hemic neoplasia positive (North River) and 40 hemic neoplasia negative (Havre-aux-Maison) clams as classified by flow cytometry was collected 5 days after the flow cytometer assessment. This allowed the clams to recover from the initial sampling. This recovery time frame was determined from previous studies carried out in our laboratory (unpublished data). A hemolymph sample of 1 ml was collected and utilized for both light and electron microscopy. For light microscopy, 0.2 ml of hemolymph was transferred to 5 ml tubes containing 2.5 ml of cold anticoagulant (NaCl, 28.4 g/L; $MgSO_4 \cdot 7H_20$, 8.7 g/L; $MgCl_2 \cdot H_20$, 5.5 g/L; $CaCl2 \cdot 2H_2O$, 1.5 g/L; KCl, 0.7 g/L, Tween 80, 0.5 ml/l L; 37-40% formaldehyde solution, 1.25 ml/L, pH 7.6) (Battison et al., 2003). The samples were then vortexed briefly and held on ice. This sample was utilized for total and differential hemocyte counts (described in sections 3.2.3.1.1 and 3.2.3.1.2). Classification of hemocytes was based on the presence or absence of granules, size, color, nuclear and cytoplasmic appearance, nucleus to cytoplasm ratios and nuclear chromatin pattern.

3.2.3.1.1 Total hemocyte count (THC)

Total hemocyte counts (the number of cells per ml hemolymph) were performed using a Neubauer haemocytometer. To do this, 20 µl of hemolymph was added to the haemocytometer chambers and examined with an Axio Imager Zeiss Microscope (40x). Hemocytes were counted in both grids (0.1 $mm^3$) and the mean value was used to calculate the THC, using the following formula: Total hemocyte count = total x dilution $x10^3$/ 0.4 = # / ml.

3.2.3.1.2 Differential hemocyte count (DHC)

For differential hemocyte counts (cell type %), 100 µl of each sample was cytocentrifuged (Cytospin 2, Shandon, Pittsburgh, USA) at 72.4g for 3 minutes. The slides were quickly and thoroughly dried using a hand-held blow dryer, then stained with a modified Wright Giemsa stain (Hema-Tek Stain Pak 4405, Bayer, Diagnostic Division, Elkhart, USA) on an automated stainer (Hema Tek 2000, Bayer Diagnostic, Elkhart, USA). Slides were mounted with Permmount media and cover-slipped. Differential hemocyte counts were performed using an Axio Imager Zeiss Microscope and oil immersion lens (100x). Slides were scanned side to side to not overlap viewing areas. One hundred cells were counted, measured and classified in areas that were evenly stained. Any cell that was lysed or disintegrated (swollen nucleus or breaks in cell outline) was excluded. Samples that had more than 10% of such cells were considered unreliable. Hemocyte measurements were taken with the Axio Imager measurement software. Nuclear and cell diameter of each hemocyte was measured. Nuclear diameter was divided by the hemocyte length to determine the N:C ratio.

Absolute counts were calculated for each hemocyte type, using this formula: Absolute hemocyte count = differential cell count (percentage of each cell type) x total hemocyte count.

3.2.4 Transmission electron microscopy (TEM)

For TEM, 4 negative (Havre-aux-Maison) and 8 positive (North River) clams classified by flow cytometry were analyzed. A volume of 0.5 ml of hemolymph was mixed with 0.5 ml of 6% cold glutaraldehyde (Canemco, Quebec) in Instant Ocean (Aquariumsystem, Mentor Iowa) (30g /1000 ml). Samples were mixed in double strength seawater (60 ppm) at 4°C overnight. These samples were then spun for 10 minutes at 635g, washed in seawater twice and postfixed in 1% osmium tetroxide for 1 hour in the same buffer. After removing the supernatant, the cell pellets were embedded in 4% agar and processed. Samples were dehydrated twice in a graded series of ethanol solutions for 10 minutes each, then placed into 70% propylene oxide and 30% resin for 2 hours at room temperature. Next, the samples were placed in a 50:50 solution of propylene oxide and resin for 2 hours and then placed in 100% resin for 24 hours in a vacuum chamber. The samples were then infiltrated in propylene oxide and Epon/Araldite mixture in 100% resin at 70°C for 2 hours. Blocks were cut on an ultramicrotome Reichert-Jung Ultracut E (Leica, Austria). Thick sections (0.5 μm) were placed on glass slides and stained with 1% Toluidine blue in 1% tetraboric acid. Thin sections (70-80 nm) were cut and placed on copper grids before staining with 5% uranyl acetate in 50% ethanol and lead Sato (citrate/nitrate mixture) stain. A Hitachi 7500 (Nissei Sangyo Inc. Quebec) transmission electron microscope at 80Kv was used to

obtain images, which were captured on a digital camera XR40 (AMTXR40). Negative images were also taken with Kodak electron microfilm 4489.

### 3.2.5 Histology

For histological analysis, 10 negative (Havre-aux-Maison) and 20 positive (North River) clams, classified by flow cytometry, were prepared. Positive clams were selected at various levels of disease intensity as determined by flow cytometry. Clams were shucked and a sagittal 3 mm thick section containing gills, visceral mass, digestive gland, foot and mantle lobes was excised, placed in cassettes and fixed in 10% seawater formalin fixative. Sections were paraffin embedded, placed on microscope slides, dried for 20 minutes at 56°C and stained with Mayers Hematoxylin and Eosin (Springer-Aldrich Inc, St. Louis, USA). The procedure used to evaluate hemic neoplasia for these slides was a qualitative one that focused on the presence or absence of neoplastic cells and their estimated percentage of total tissue cells in various organs. The following scale was used: Light - <20% neoplastic cells, Moderate - >20% - <50% neoplastic cells, Heavy - >50% neoplastic cells.

## 3.3 Results

### 3.3.1 Flow cytometry

Forward angle scatter (FSC) provides a relative indication of the cell size, while side angle scatter (SSC) provides an indication of complexity, texture or granularity of cells. Both parameters revealed one large group of hemocytes from presumed negative and

positive clams on a scatterplot. Discriminating between heterogenous hemocyte populations along the FSC and SSC axis was not possible.

3.3.2 Hemocyte morphology - light microscopy

Five types of hemocytes in hemic neoplasia negative clams were distinguished using light microscopy. The mean (±SD) nuclear diameters, cell diameters and N:C ratios of the different hemocyte types for both populations are shown in Table 1. The following hemocyte types in the soft-shell clam identified were:

(1) **Type 1 granulocyte**: This cell type had a round basophilic eccentrically located nucleus with a mean diameter of 4.2 μm (±0.95). The nucleus had a moderately clumped chromatin pattern. The N:C ratio was low. Cells had a mean width of 11.8 μm (±1.07) and mean length of 10.9 μm (±0.9). These cells contained a high density of small (<1 μm) eosinophilic granules, distributed throughout the cytoplasm (Figure 1a).

(2) **Type 2 granulocyte**: This cell type had an oval eosinophilic central to slightly eccentric nucleus with a mean diameter of 5.5 μm (±0.60). The nucleus had a slightly clumped chromatin pattern. The N:C ratio was moderate. Cells had a mean width of 10.3 μm (±0.83) and mean length of 9.2 μm (±1.05). These cells contained low numbers of small (< 1μm) eosinophilic granules in the cytoplasm (Figure 1b).

(3) **Type 1 agranulocyte**: This cell type had a round basophilic eccentric nucleus with a mean diameter of 4.8 μm (±0.70). The N:C ratio was low. Cells had a mean width of 11.6 μm (±1.0) and a mean length of 10.6 μm (±0.9). These cells lacked granules, but numerous clear round vacuoles varying in size from approximately 0.5 μm to 1.5 μm were present in the cytoplasm (Figure 1c).

(4) **Type 2 agranulocyte**: This cell type had a slightly oval basophilic eccentrically located nucleus with a mean diameter of 5.6 μm (±0.45). The nucleus had a slightly coarse chromatin pattern. The N:C ratio was moderate. Cells had a mean width of 10.1μm (±0.90) and mean length of 8.8 μm (±1.4). These cells lacked granules (Figure 1d).

(5) **Type 3 agranulocyte**: This cell type had an oval dark basophilic centrally located nucleus with a mean diameter of 3.9 μm (±0.54). The nucleus had a coarse clumped chromatin pattern. The N:C ratio was high. Cells had a mean width of 6.25 μm (±0.67) and mean length of 6.18 μm (±0.76). These cells lacked granules (Figure 1e).

The mean (±SD) total hemocyte count for the negative clams was $4.7 \times 10^6$ ($\pm 4.8 \times 10^5$) cells ml $^{-1}$ of hemolymph. The mean (±SD) total hemocyte count for the positive clams was $4.6 \times 10^6$ ($\pm 9.1 \times 10^5$) cells ml $^{-1}$ of hemolymph. The mean differential counts for the granular and agranular cells of the negative clams was determined. To do this, the 2 types of granulated and 3 types of agranular cells were combined into granulated and

agranular cell categories, respectively. This was an arbitrary decision, as it was recognized that these cells may represent different maturation stages or de- granulated cells. The mean (±SD) differential hemocyte count for the negative clams (Havre-aux-Maison site) was 62.4% (± 11.2) agranular and 34.9% (± 10.8) granular cells. Differential hemocyte counts for negative clams are summarized in Table 2 (Appendix C1) with absolute counts summarized in Table 3 (Appendix C1). Differential hemocyte counts for the positive clams are summarized in Table 4 (Appendix C2) with absolute counts summarized in Table 5 (Appendix C2). A lower percentage of agranular cells (Table 6: Appendix C2) was observed in positive clams with >50% 4N cells compared to positive clams with <50% 4N cells.

**Neoplastic cells**: Two types of neoplastic cells were identified. Type A cells had a lobulated, pleomorphic appearance with basophilic nuclei, a stippled chromatin pattern and frequent nuclear clefts. Nucleoli ranged from single to multiple in approximately 10% of Type A cells and were not observed in the remaining 90%. These cells lacked granules in the cytoplasm but vacuoles were occasionally observed (Figure 2a). Type B cells had round basophilic nuclei, a coarse dispersed chromatin pattern and distinct nuclear borders. These cells had a more uniform appearance than the type A cells. Single nucleoli were observed in approximately 5% Type B cells. Nucleoli were not observed in the remaining 95% (Figure 2b). The mean nuclear diameter (± SD), mean width (± SD) and mean length (± SD) of the different types of neoplastic cells are summarized in Table 1.

**Table 1**: Mean (±SD) nuclear diameters (μm), cell diameters (μm) and their ratio (N/C) in *Mya arenaria* hemocytes from North River, PEI and Havre-aux-Maisons, QC.

| Parameter | Type 1 Granular | Type 2 Granular | Type 1 Agranular | Type 2 Agranular | Type 3 Agranular | Type A neoplastic | Type B neoplastic | Combined neoplastic |
|---|---|---|---|---|---|---|---|---|
| Cell length | 10.9 (±0.9) | 9.2 (±1.05) | 10.6 (±0.9) | 8.8 (±1.4) | 6.18 (±0.76) | 11.9 (±1.6) | 9.4 (± 1.0) | 10.9 (±1.8) |
| Cell width | 11.8 (±1.07) | 10.3 (±0.83) | 11.6 (±1.0) | 10.1 (±0.90) | 6.25 (±0.67) | 13 (± 1.5) | 10 (± 1.2) | 1.7 (±1.9) |
| Nuclear diameter | 4.2 (±0.95) | 5.5 (±0.60) | 4.8 (±0.70) | 5.6 (±0.45) | 3.9 (±0.54) | 9.9 (± 1.1) | 8.8 (± 0.8) | 9.4 (±1.1) |
| N/C ratio | 0.38 | 0.59 | 0.45 | 0.63 | 0.75 | 0.83 | 0.93 | 0.83 |

n = 80 clams (8000 cells)

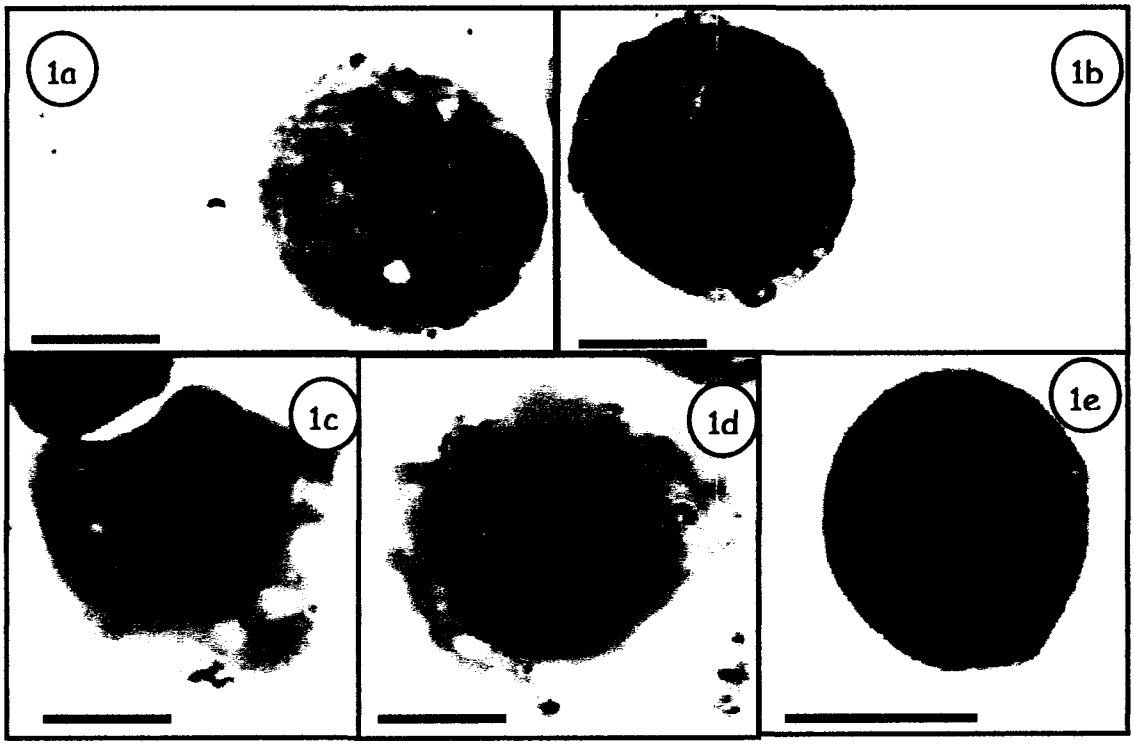


**Figure 1:** Light micrographs of hemocytes from soft-shell clams stained with modified Wright Giemsa. (a) Type 1 granulocyte containing numerous granules (63x) (b) Type 2 granulocyte containing a moderate number of granules (63x) (c) Type 1 agranulocyte (63x) (d) Type 2 agranulocyte (63x) (e) Type 3 agranulocyte (63 x 1.6) scale bar: 20 μm

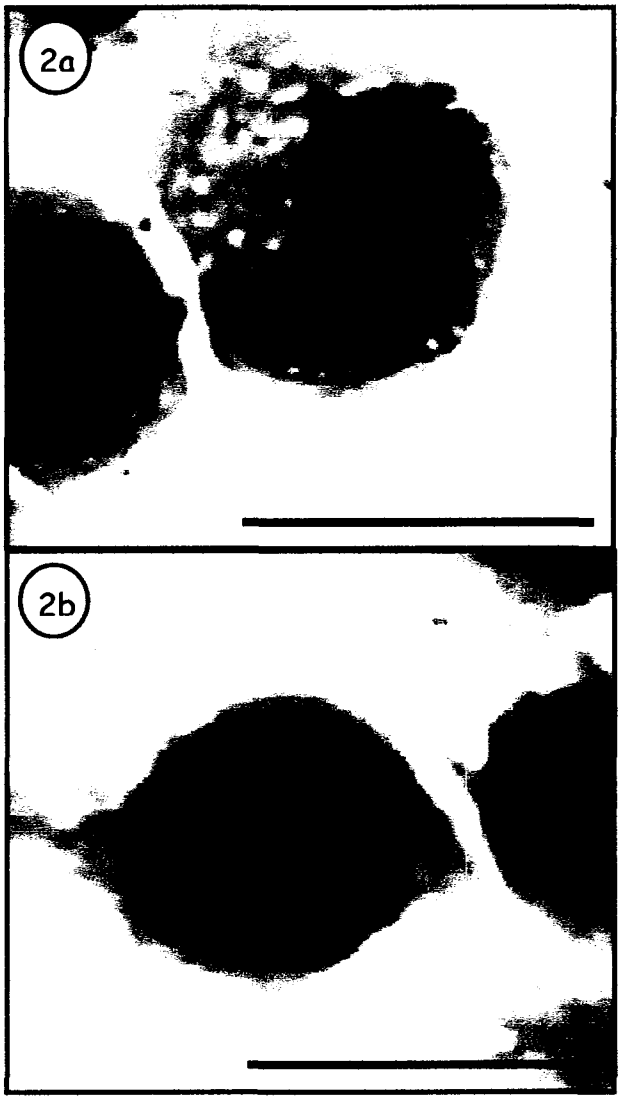


**Figure 2:** Cytospin prepared slides of *Mya arenaria* neoplastic cells. Slides stained with modified Wright Giemsa **(a)** Type A neoplastic cell **(b)** Type B neoplastic cell (slides viewed at 100x: scale bar: 20 μm)

3.3.3 Hemocyte morphology- transmission electron microscopy

The criteria for recognizing hemocytes with light microscopy were applied for transmission electron microscopy.

(1) **Type 1 granulocyte**: The nucleus of these cells had euchromatin (lightly stained chromatin) with heterochromatin (darkly stained chromatin usually seen in the periphery of nucleus) dispersed as clumps and marginated along the border. Numerous spherical to reniform electron dense, membrane bound granules ranging in size from 0.2-0.8 μm in diameter were observed. The granules were composed of heterogeneous electron dense granular matrix. These granules were found scattered throughout the abundant cytoplasm. The cytoplasm contained numerous electron lucent zones and circular vesicular bodies. Irregularly shaped mitochondria, rough endoplasmic reticulum and Golgi apparatus were observed in the cytoplasm (Figure 3a).

(2) **Type 2 granulocyte**: The nucleus of these cells contained moderate amounts of both euchromatin and coarse clumps of heterochromatin. The cytoplasm contained small numbers of spherical to reniform electron dense membrane bound granules ranging in size from 0.2-0.5 μm in diameter. The granules were composed of a heterogeneous electron dense granular matrix. The cytoplasm contained electron lucent zones and circular vesicular bodies. The Golgi apparatus, irregular shaped mitochondria and profiles of rough endoplasmic reticulum were observed in the cytoplasm. Ribosomes were present in very high numbers in this cell type (Figure 3b).

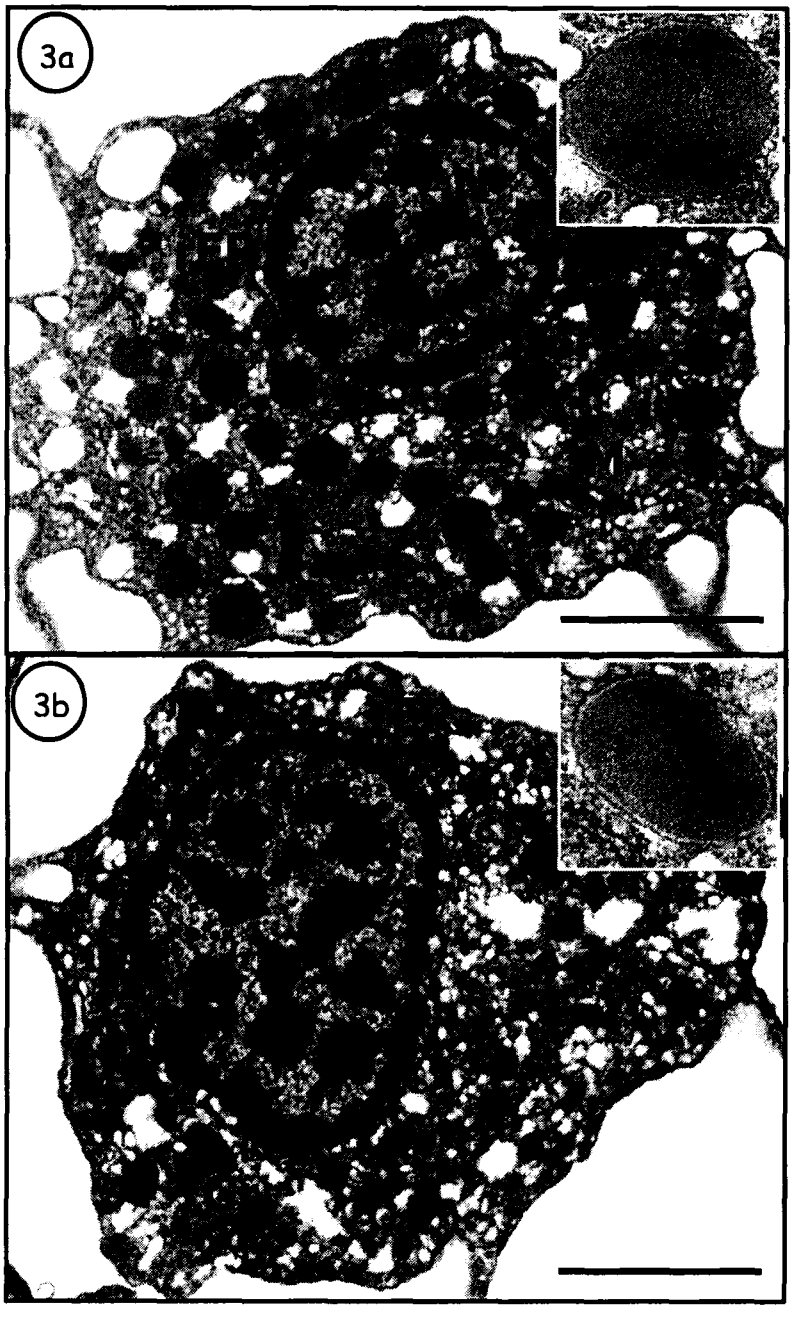


**Figure 3**: Transmission electron micrographs of granulocytes of healthy soft-shell clams (a) Type 1 granulocyte containing numerous large membrane bound electron dense granules (20,000x). Insert: high magnification of granule (80,000x). (b) Type 2 granulocyte containing few membrane bound electron dense granules (20,000x). Insert: high magnification of granules (80,000x). Scale bars: 2 μm. N - nucleus; Gr - granule; rER - rough endoplasmic reticulum; M - mitochondria.

(3) **Type 1 agranulocyte**: The nucleus of these cells contained moderate amounts of euchromatin and heterochromatin with thick margination of heterochromatin along the border. Considerable amounts of circular vesicular bodies were seen in these cells. Rough endoplasmic reticulum, ribosomes, mitochondria and prominent Golgi apparatus were also observed. Possible glycogen deposits measuring 30-35 nm were observed in the cytoplasm. Granules were not observed in the cytoplasm (Figure 4a).

(4) **Type 2 agranulocyte**: The nucleus of these cells contained a moderate amount of euchromatin and coarse clumps of heterochromatin. Rough endoplasmic reticulum and small spherical mitochondria were observed in the cytoplasm. Possible glycogen deposits were observed in the cytoplasm (Figure 4b).

(5) **Type 3 agranulocyte**: This small cell type contained a large spherical nucleus and scant cytoplasm. The nucleus of these cells contained a large amount of coarse clumped heterochromatin. The cytoplasm of these cells lacked most organelles except long profiles of rough endoplasmic reticulum and numerous mitochondria (Figure 4c).

**Neoplastic cells**: Type A neoplastic cells were recognized by their large size, large nucleus and pleomorphic features. The nucleus contained a stippled chromatin pattern with abundant amount of euchromatin. The nuclear border of these cells was irregular, contained macro-clefts and was indistinct. These cells lacked most organelles except for numerous mitochondria surrounding the nucleus.

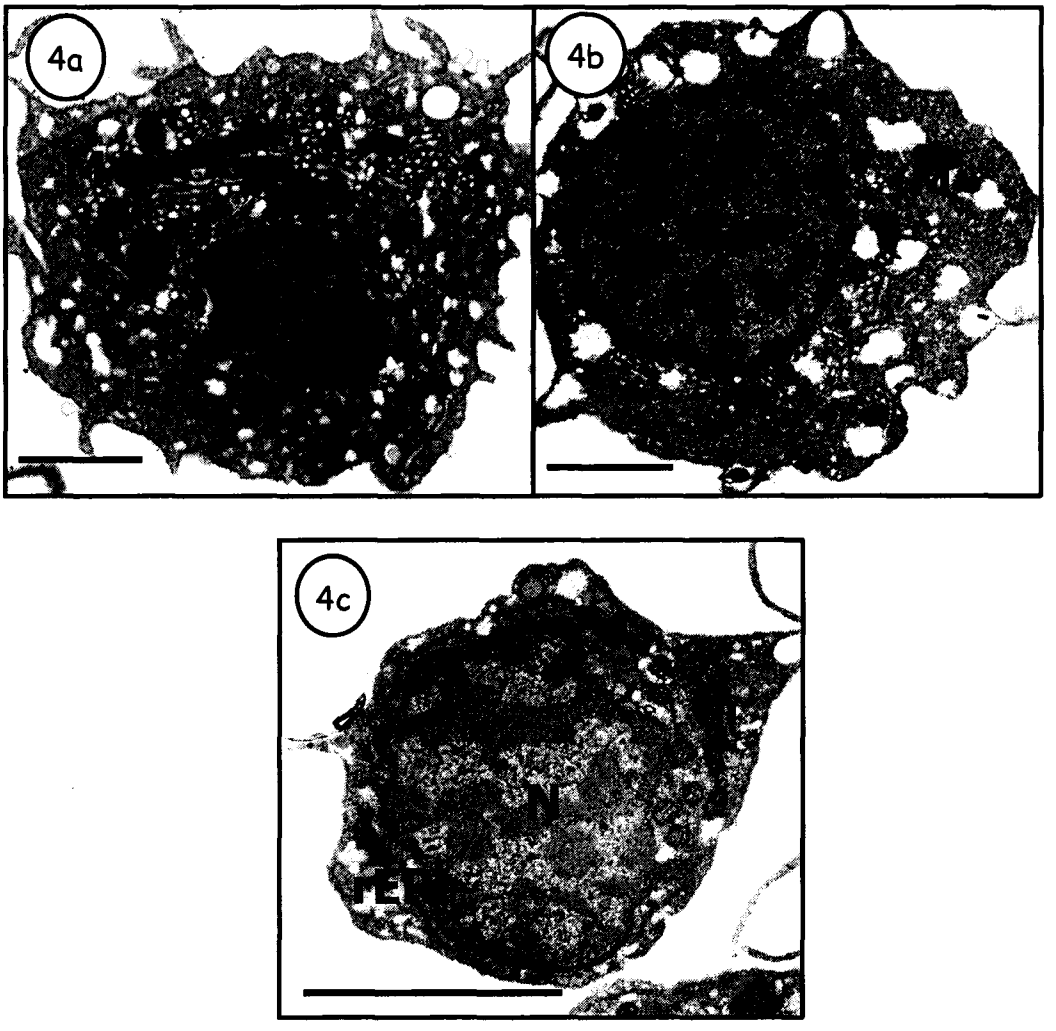


**Figure 4**. Transmission electron micrographs of *Mya arenaria* agranulocytes from healthy clams. (a) Type 1 agranulocyte (20,000x) (b) Type 2 agranulocyte (20,000x) (c) Type 3 agranulocyte (20,000x) Scale bars – 2 μm. N – nucleus; M - mitochrondria; rER – rough endoplasmic reticulum.

These cells retained few pseudopodia extensions (Figure 5a). Type B neoplastic cells were smaller than Type A and contained a large ovoid nucleus with a dispersed chromatin pattern. A single nucleolus was frequently seen in these cells. The nuclear border was irregular and displayed heterochomatin margination. The cytoplasm of these cells was very scant and lacked organelles (Figure 5b). Some cells (< 20% of total number) had features of both Type A and B and may represent an intermediate form. Unique features of these cells included a nucleus with loss of heterochromatin, abundant small circular mitochondria. Bi-nucleate cells were commonly observed (Figure 5c).

### 3.3.4 Histology

Mitotic figures were observed in the gills of one negative clam. For the hemic neoplasia positive individuals, clams were divided into light to moderately positive (<50 % 4N cells) and heavily positive (> 50% 4N cells) groups, as determined by flow cytometry (Table 7 Appendix D1). In the light to moderately positive group, neoplastic cells were observed predominately in the gills and digestive gland. In the heavily positive group, neoplastic cells were observed in the gills, mantle, gonad, digestive gland, kidney and foot. Mitotic figures were found in 3 clams in this group, specifically in the mantle and foot. Neoplastic cells were found infiltrating the sinuses in 4 clams in this group. Loss of tissue architecture was observed in a few clams with advanced neoplasia. A Rickettsia - like organism was observed in the digestive gland of 2 clams in the heavily positive group. Figure 5 (a-b) (Appendix D2) provide images of negative and positive clams.

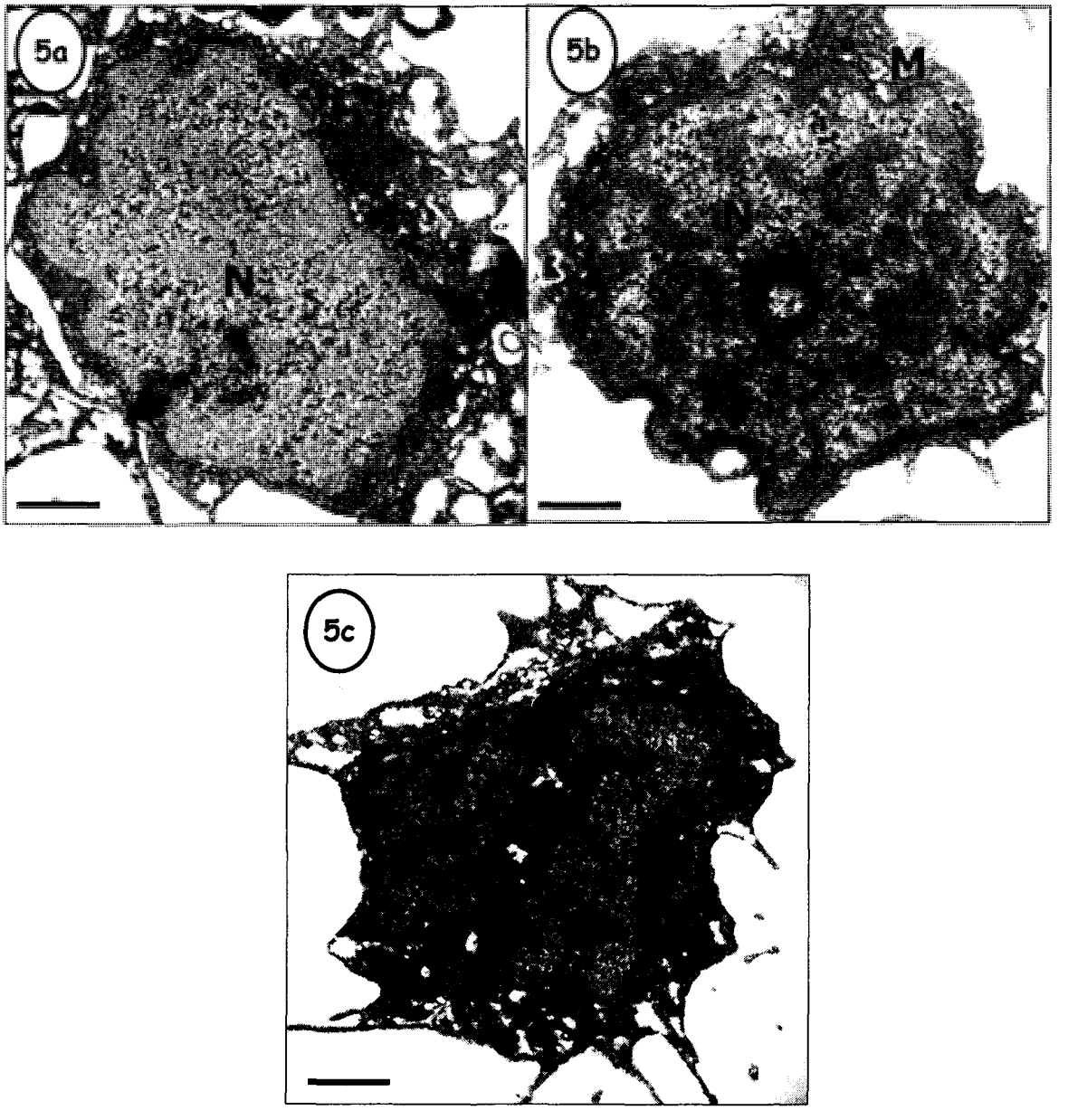


**Figure 5**. Transmission electron micrographs of *Mya arenaria* neoplastic cells from hemic neoplasia positive clams (a) Type A neoplastic cell (20,000x) (b) Type B neoplastic cell (20,000x) (c) Binucleated cell (20,000x) Scale bars – 2 μm. N – nucleus; M - mitochrondria.

### 3.5 Discussion

Sampling clams from two geographically different populations may have resulted in bias. Population variation resulting from genetic differences between populations, environmental differences and physiology may affect the number and types of hemocytes in bivalves (Cao et al., 2007). Due to this variablity and the fact that the populations were sampled at different times during the year, the total and absolute differential counts were not statistically compared in this study. These counts were established to contribute to the literature values for normal clams and clams with presumed hemic neoplasia.

Hemic neoplasia has been documented at various levels of disease intensity and prevalence from several soft-shell clam populations in Atlantic Canada (McGladdery et al., 2001). The Magdalen Islands population has historically been documented as having low disease intensity and disease prevalence. Nevertheless, uncertainties remain because of phenotype reliance. Even though the case definition for individual clams deemed positive for hemic neoplasia was determined to be >5% 4N, (Chapter 2), this is an arbitrary threshold that will change depending on the objectives of the study.
For this study, characterizing normal hemocytes from clams strictly negative for hemic neoplasia were required. Therefore, it was important to decrease the threshold. Decreasing the threshold to <1% 4N increases the sensitivity of the diagnostic test to try and ensure only negative clams are selected. Because the North River population has high disease intensity and prevalence, clams diagnosed as negative from this site were not judged to be good candidates for characterizing the normal morphological features

of hemocytes. This is because clams assessed as negative from this population may have a higher chance of being in the early stages of neoplastic transformation than those classified as negative from a population with low disease intensity and prevalence. Hemocytes from clams with a low percentage of 4N cells may still be at an early stage of neoplastic transformation but may not yet be expressing an abnormal ploidy.

Total and differential white blood cell counts in human and veterinary medicine is used in routine diagnostic procedure to identify stress, inflammation or other conditions. The use of established cellular reference intervals are used to provide guidelines for identifying normal and abnormal situations. Since mammals have a closed circulatory system, maintaining a steady state in their internal environment is an important aspect of homeostasis. In this regard, these animals are regulators. Evaluating the total and differential hemocyte counts in bivalves is more complicated. Bivalves are non-homeostatic animals and they conform to changes in their environment (Cao et al., 2007). Seasonal parameters (temperature, salinity, food availability) environmental parameters (water depth, sediment, anthropic activities) and physiology (gametogenesis) cause variation in the total and differential hemocyte counts (Pipe, 1997; Oliver et al., 2005). Carballal et al. (1998) reported that the maximum number of hemocytes in the mussel, *Mytilus galloprovincialis,* occurs during the summer months, peaking in July. These authors suggest food availability and gametogenesis as factors. Pampanin et al. (2002) also reported the highest total hemocyte count in the summer months, compared to spring and winter in the Venus clam, *Chamelea gallina*. Contrary

to these studies, Santarem et al. (1994) reported total hemocyte count as the lowest during the summer months in *M. galloprovincialis*.

These studies reflect the level of numerical hemocyte variation that is commonly observed in bivalves of different populations and from different environments. Reliance on the total hemocyte count can be misleading if these factors are not taken into consideration when interpreting results. A reference interval for the total hemocyte count would have to be defined for multiple environments with multiple yearly sampling, resulting in several reference intervals. Interpretation of several indicators is complicated and as a result, most investigators continue to rely only on the total hemocyte count as a rapid non-lethal assessment.

In the present study, there was larger total hemocyte count variability in the hemic neoplasia positive group (range: 3.5 - 7.5 $x10^6$ ml) compared to the hemic neoplasia negative group (3.3 - 5.5 $x10^6$ ml). The positive clams ranged in disease intensity from a very low level to very high. Clams with higher disease intensity had higher total hemocyte counts. Compared to negative clams, hemic neoplasia positive clams have been reported to have higher total hemocyte counts (Stephens et al., 2001). Several papers refer to these neoplastic cells as overcrowding the healthy hemocytes, resulting in death as these cells compete for oxygen and nutrients (Farley, 1969 and 1986; Cooper et al., 1982; Peters, 1988; Sinderman, 1990; Elston et al., 1992).

In the present study, 5 types of hemocytes were recognized in the soft-shell clam. The criteria used to recognize these hemocytes included the presence or absence of granules,

cell size, staining affinity, nucleus position and N:C ratio. These criteria are similarly applied in mammal cell studies. The morphology of precursor cells in mammals changes as the cells go through maturation steps. Morphologically, these steps involve changes in the size of the cell, chromatin pattern, color affinity, presence or absence of nucleoli and nucleus position (Kumar et al., 2005).

Hematopoiesis and the differential pathways of maturing hemocytes are unknown in bivalves. Classifying cells when there is a morphologic continuum can be challenging. Several studies examining hemocyte populations suggest that hemocytes are a mixture of different subpopulations of cells. Mix (1976) suggested the one cell theory whereby agranular cells represent a proliferative stage that become granulocytes when mature. Cheng (1981) suggested that granulocytes and agranulocytes represent two distinct cell lineages. In fact, there is no evidence to support that different cell lineages exist in bivalves. Unlike vertebrates, bivalve hemocytes are less specialized and have multiple roles in nutrition, excretion and defense (Hine, 1999). Hemocytes may display different morphologic features depending on their maturation stage or functional role at the time of sampling. Thus, these hemocytes may represent one cell lineage like the one Mix (1976) proposed. In the present study, granular cells were separated into 2 types based on staining affinity, nucleus position and number of granules. However, it is possible that the two granulated cells represent different developmental stages. It is also possible that the differential granulation reflects intact and degranulated forms of the same hemocyte group.

In the present study, clams were sampled during the spring and had an overall mean percentage of 62.6% agranulocytes and 34.7% granulocytes. Huffman and Trip (1982) reported an overall mean percentage of 23.5% agranulocytes and 76.5% granulocytes in the soft-shell clam during winter sampling. Variability in granulocyte and agranulocyte subpopulation percentages may be a result of different geographical populations and seasons. More sophisticated studies are required to aid in the bivalve hemocyte categorization. For example, the level of hemocyte differentiation in the soft-shell clam could be analyzed further with lectin binding and enzymatic studies. In addition, the expression of surface markers on the cell membranes could be used to evaluate the possibility of different cell lineages.

Vertebrate blood cells develop from pluripotential stem cells that develop into monopotential specialized cells representing different cell lineages (Kumar et al., 2005). It is unknown if stem cells exist in bivalves. Cima et al. (2000) described a cell they referred to as a hemoblast in the Manila clam, *Ruditapes philippinarum*. They described the hemoblast as small and undifferentiated, approximately 3-5 μm in diameter, with a high N:C ratio and no enzyme activity. Similar studies on the Venus clam, *Chamelea gallina*, and hard clam, *Meretrix lusoria*, describe blast like hemocytes with a deeply basophilic nucleus and a high N:C ratio (Pampanin et al., 2002, Chang et al., 2005). This cell is similar to the small type 3 agranular cell observed in the present study. However, without further evidence it is speculative as to whether these are the same cell type as reported previously.

Malignant tumors are characterized by transformation of the affected cell, rapid growth of these transformed cells, invasion and distant metastasis (Kumar et al., 2005). A common term used to describe the behavior of these cells is anaplasia (lack of differentiation). Features of anaplasia include pleomorphism, abnormal nuclear morphology, bi-nucleate cells, mitotic figures and loss of polarity (Kumar et al., 2005). Literature descriptions of neoplastic cells in bivlaves with hemic neoplasia describe them as large round anaplastic appearing cells approximately 10-20 μm in diameter (Miosky et al., 1989; Moore et al., 1992) with a high N:C ratio, one or more nucleoli and frequent mitosis (Yevich and Barszcz, 1976 and 1977; Lowe and Moore, 1979). Several investigators support the idea that these neoplastic cells are hemocytic in origin in bivalves (Elston, 1990; Barber, 2004). Tissue distribution, together with immunocytological evidence (Reinisch et al., 1983; Smolowitz et al., 1989) suggest that the neoplastic cells are hemocytes. However, several investigators use the more conservative term of disseminated neoplasia to describe this condition until further information on the embryological and developmental origin of these neoplastic cells is established.

In the present study, compared to non-neoplastic cells, presumed neoplastic cells were larger with a high N:C ratio, abnormal nuclear morphology (hyperchromasia, presence of single or multiple nucleoli), and a pleomorphic appearance. The presence of bi-nucleated cells and mitotic figures were frequently seen at various levels of disease intensity. These cells also seemed to have fewer pseudopodia extensions compared to non-neoplastic cells. Two types of transformed cells were recognized. Possible

intermediate stages, sharing characteristics of both, were also observed. Ultrastructurally, abundant euchromatin and mitochondria were observed in these neoplastic cells indicating increased activity.

The level of cellular transformation of the neoplastic cells may offer insight into the ability of these cells to infiltrate tissue. Histology provided information on the movement and quantity of transformed cells in the tissues and organs at various levels of disease intensity. However, it was not possible to distinguish between the different types of neoplastic cells; this requires further work. Large basophilic cells with high N:C ratios were observed in the gills and digestive gland in clams with low disease intensity. Normal hemocytes are usually observed in these areas in healthy clams. In more severe situations of the disease, neoplastic cells were observed infiltrating the gills, mantle, gonad, digestive diverticula and kidney. Hemocytes are normally found in these organs in healthy clams but to a lesser degree. Neoplastic cells were also observed in the foot. Hemocytes are rarely seen in the interior of muscle bundles of the foot in healthy clams. Caution must be exercised when referring to tissue invasion in bivalves due to the open circulatory system. Following the invasion of neoplastic cells into various tissues can be difficult because of uncertainties of tissue penetration. Special cytochemical stains for cell differentiation and monoclonal antibodies specific for neoplastic cells are useful.

Previous observations support the potential for two types of neoplastic cells in bivalves. Brown et al. (1971) described two types of neoplastic cells in the soft-shell clam. They

consisted of large cells with little cytoplasm and multiple nucleoli and intermediate sized round cells with one prominent nucleolus. Similar observations exist for the American oyster, *Crassostrea virginica,* the Olympia oyster, *Ostrea lurida* (Farley and Sparks, 1970) and blue mussel, *Mytilus edulis* (Lowe and Moore, 1978; Green and Alderman, 1983). Despite previous descriptions of two types of neoplastic cells in the soft-shell clam, these observations have not received a great deal of attention.

The majority of studies utilize clams in advanced stages of hemic neoplasia to identify antigen and protein expression or dysregulation of genes (Reinisch et al., 1983; Smolowitz and Reinisch., 1986; Miosky et al., 1989; Stephens et al., 2001; Walker et al., 2006). In the present study, an association was not observed between the type of neoplastic cell observed microscopically and the amount of 4N cells determined by flow cytometry. Both types of neoplastic cell were present at various levels of 4N cell presence. Further work with cell markers may assist in understanding disease progression. The level of transformation may coincide with the expression of antigens and dysregulation of genes contributing to the pathogenesis of hemic neoplasia. For example, Type A or B neoplastic cells may be at earlier or later stages of transformation. One type may not express surface antigens identifiable by Mab 1E10 (specific for neoplastic cells) whereas a cell at a later stage of transformation might. Likewise, the dysregulation of tumor suppressor genes such as p53 may occur at an earlier stage of disease, causing the cells to proliferate out of control with a potentially altered morphology.

The occurrence of two types of neoplastic cells in bivalves may be a reflection of a heterogeneous population of cells originating from one cell lineage, referred to as a single cell lineage or clonal hypothesis. According to this hypothesis, a single morphologically transformed cell replicates and produces other fully transformed cells. Studies have shown there is an extensive degree of variability between these cells accounting for differences in their ability to progress (Merlo et al., 2006). Hemic neoplasia may alter any stage of granular or agranular development; no conclusion can be made at present. Past investigators have suggested that hemic neoplasia is from an agranulocyte lineage (Barry et al., 1971; Lowe and Moore, 1978; Farley et al., 1986; Elston et al., 1995). Recognizing neoplastic cells from agranular cells (type 3) was occasionally challenging in the present study. Type 3 agranular cells were smaller than presumed neoplastic cells, but had a high N:C ratio with scant cytoplasm and nuclear hyperchromasia. The use of special stains or RNA probes in the future may help distinguish between these cells. A decrease in the percentage of agranular hemocytes was observed in clams diagnosed with more then 50% 4N cells in the present study. This may be because the agranular hemocyte cell line has become neoplastic or it may be that the neoplastic cells are of a different cell line. If the latter is true, the neoplastic cells may be sending inhibitory signals to the agranular cell line. This situation of a neoplastic cell population inhibiting proliferation of another cell line is well recognized in humans (Kumar et al., 2005). Further studies are required to determine if this shift in hemocyte types is due to neoplastic transformation or an inhibitory phenomenon.

# Chapter 4

# General Discussion

## 4.1 Introduction

The soft- shell clam has been a staple of the Atlantic Canadian seafood market for many decades. In 1993, there were three clam growers in PEI, which expanded to 16 growers in 1999 (MacNair and Smith, personal communication). However, mass mortalities of soft-shell clams caused by or associated with hemic neoplasia occurred at several sites in PEI and New Brunswick in 1999 (McGladdery et al., 2001). Since this time, the clam industry has struggled in Atlantic Canada and there are currently only 2 growers remaining in PEI.

## 4.2 Hemic neoplasia disease classification

Traditional methods for diagnosing hemic neoplasia have relied on cytology and histology for recognizing morphological characteristics of abnormal cells. Although microscopic techniques provide a visual examination and permanent record, these techniques are subjective and labor intensive. In order to improve this situation and increase diagnostic sensitivity, techniques that are reliable and non-lethal are required. Flow cytometry meets these criteria and offers a quantitative assessment of hemic neoplasia. Flow cytometry demonstrated its potential in the present study by objectively separating positive and negative individuals and populations according to arbitrarily assigned ploidy thresholds. Flow cytometry allows rapid ploidy assessment of thousands of cells per second from a small volume of hemolymph. The ability to screen large numbers of cells greatly exceeds the capacity of traditional microscopy

methods. Unlike previous studies in Atlantic Canada that have relied on qualitative microscopic techniques (McGladdery et al., 2001; McGladdery and Davidson, 2003), flow cytometry provides a more accurate quantitative examination of hemocytes.

Reno et al. (1994) performed a flow cytometric and chromosome study which provided support that hemic neoplasia in the soft-shell clam is characterized by tetraploidy. Similar studies on *Mytilus edulis* showed that neoplasia is recognized by tetraploid and/or pentaploid DNA content (Elston, 1990; Moore and Lowe, 1991). Tetraploidy is not specific to neoplasia. It has been suggested that in normal clams, a small percentage of hemocytes extend their time in the S phase and are tetraploid (Reno et al., 1994). Reno et al. (1994) suggested that clams without neoplasia could have approximately 4-6% of total hemocytes as tetraploid. However, further studies are required that focus on clam physiology under different environmental conditions. A slight increase in DNA content may not necessarily mean the clam has hemic neoplasia, but may reflect variations in physiology, hyperplasia or dysplasia. This may explain the supposed remission phenomenon for neoplasia in clams (Barber, 1990; Brousseau and Baglivo, 1991). These studies suggest that clams originally diagnosed using cytology as having "low level" hemic neoplasia reverted to negative status. However, these studies do not provide a case definition for negative and positive clams, and it is possible that the low-level status may have instead been due to a non-neoplastic process. Cases of spontaneous remission of neoplasia are extremely rare in humans and mammals. Therefore, unless clams possess an unusual neoplasia reversal capability, a situation in which a proliferative response normalizes is highly unlikely to be neoplastic.

In the present study, differentiating neoplastic versus non-neoplastic situations was difficult when the data displayed a normal 2N peak in the $G_o/G_1$ phase of the cell cycle but a slightly abnormal increase in 4N cells in the S phase. Due to uncertainty in diagnosing these clams as negative or neoplastic, a threshold was developed. Although it was anticipated that false positive and negatives would exist, a percentage cut-off for hemocytes with 4N ploidy provided a case definition to assign clams to positive and negative groups, which has not been done prior to this study. To help confirm the diagnosis of neoplasia versus non-neoplasia, cytology was done in an initial attempt to classify hemocytes as neoplastic and to offer a visual examination not possible with flow cytometry. However, challenges arose with the use of cytology, as cells in the early stages of hemic neoplasia were not confidently identified. Ambiguities regarding hemocyte characteristics (both normal and neoplastic) led to difficulties in diagnosing clams as disease positive and negative. These ambiguities resulted from a lack of information on the types of hemocytes, functions and cell lineages in the soft-shell clam. Over the years, investigators have described the morphology and behavior of neoplastic cells without a clear description of the normal hemocytes. Consequently, the morphological criteria identifying a neoplastic hemocyte in bivalves is vague. The majority of studies rely on limited phenotypic information for recognizing neoplastic cells, such as a round shape and inability to adhere to glass (Miosky et al., 1989; Leavitt et al., 1990; Beckman et al., 1992; Walker et al., 2006). These criteria are extremely weak compared to the robust criteria applied in mammalian studies.

To address these concerns, a cell study was devised to describe normal and neoplastic hemocytes with light and transmission electron microscopy. Total and differential hemocyte counts were determined for each clam. Five types of hemocytes were recognized in presumably healthy clams. An understanding of the morphological characteristics of normal cells was then used to identify neoplastic cells in clams assessed as positive on flow cytometry. Some normal agranular hemocytes (type 2 and 3) displayed a moderate to high N:C ratio and were difficult to recognize as non-neoplastic at the beginning of the study. Once further experience was gained, these cells were more confidently classified as non-neoplastic; they were considered possibly a different maturation stage of a normal hemocyte. Compared to normal hemocytes, neoplastic cells appeared anaplastic. These cells were larger than normal hemocytes and had a higher N:C ratio, round or lobulated nuclei and a diffuse chromatin pattern with one or more nucleoli per cell. The presence of bi-nucleate cells and mitotic figures were also observed in positive clams. Two types of neoplastic cells and possible intermediate stages were observed at various levels of tetraploidy intensity, possibly representing one cell lineage.

Histology was also included in the present study to address two objectives. The first was to gather information on disease pathology in the tissues and organs. The second objective was to see if it was possible to recognize the two types of neoplastic cells in tissue preparations. Unlike cytospin prepared slides that provide a monolayer of readily discernable cells, it was not possible to distinguish between the type A and B neoplastic

cells using histology. The anaplastic appearing neoplastic cells that were observed were large, basophilic and overcrowded the tissues and organs.

Once the development of specific criteria for recognizing normal and neoplastic cells was established on cytology, it was applied to the population study. For cytology, a literature case definition was utilized (Farley, 1969 a and b; and Baglivo, 1991 a and b; McGladdery et al., 2001). A level of agreement between flow cytometry and cytology was determined. For the combined data of six populations, it was determined that the best agreement between both techniques was observed when a 5% 4N threshold was applied. Therefore, an individual clam was deemed to be positive for hemic neoplasia if there were greater than 5% of the hemocytes classified as tetraploids using flow cytometry. The Saguenay Bay site was the only sample with all clams having <5% tetraploid cells with the flow cytometer and < 1% neoplastic cells by cytology. Based on the mean percentage of 4N cells, this sample was determined to be significantly different ($p = <0.003$) from the other sites. Evaluation of disease intensity from a population perspective included the addition of a second threshold of >25% tetraploid cells. A population was considered positive for hemic neoplasia if more then one clam had >25% 4N cells of total hemocytes. The mean disease intensity of each population was compared and the North River site was determined to be statistically different ($p=0.003$) than all other sites. The other sites had high disease prevalence and low disease intensity. These populations may be considered positive for hemic neoplasia due to the high prevalence. However, they can remain viable, as the presence of the disease may not result in appreciable mortalities. Results like these require further studies to

determine the role that disease prevalence and intensity have on disease progression and mortality. The factors affecting prevalence and intensity may fluctuate on a seasonal basis. Thus, it is important to assess these populations on a yearly basis with multiple sampling times and large sample numbers.

### 4.3 Contribution to the study of hemic neoplasia and future recommendations

Flow cytometry provided a quantitative assessment for diagnosing hemic neoplasia and provided the ability to objectively separate positive and negative clams. This study contributes to the knowledge on the spatial distribution of hemic neoplasia, which has not received attention since McGladdery's histological survey of several sites in Atlantic Canada in 2003. The diagnostic scale of 0 - 3 stages that was developed for cytology may assist in the diagnosis of hemic neoplasia by taking into account the various levels of intensity. In addition, this scale takes into consideration the possibility that a small percentage of cells may appear abnormal in a clam that is free of hemic neoplasia. Other scales reported have a broad range of categories. For example, Farley et al. (1986) used a cytology scale that included negative clams as having 0% neoplastic cells, an early stage having 0.01 - 0.9% disease intensity, a very broad intermediate stage having 1 - 49% and an advanced stage having 50 - 100%. Brousseau and Baglivo (1991a) considered low level stage of hemic neoplasia as 1 - 50% neoplastic cells of total hemocyte counts and an advanced stage having 51 - 100%. Although useful, these categories do not consider non-neoplastic processes nor disease progression. Clams may be placed erroneously into early or advanced stages resulting in false positive results.

The use of different stages between investigators makes it difficult to assess the geographical distribution of hemic neoplasia in terms of prevalence and intensity.

The majority of studies on hemic neoplasia utilize normal clams and those with advanced neoplasia (90 - 100% intensity). By not evaluating any intermediate situations, there studies do not offer assistance in understanding disease progression. Neoplasia can be considered an evolutionary process involving multiple changes in a heterogeneous population of cells.

Apart from the description of two types of neoplastic cells by Brown et al. (1971) the present study is the only one which has focused on the morphological changes that occur to hemocytes during neoplasia. This information not only contributes to the knowledge of neoplastic cells but also provides the necessary basis for subsequent cellular and molecular investigations. As all forms of neoplastic cells were found at various levels of disease intensity in the present study, further scrutiny of the reliability of morphology and tetraploidy as indicators of hemic neoplasia is needed. It is often perceived that neoplastic cells in any species all have the same capacity to proliferate and amplify the disease, but it is now known that only a subset of neoplastic cells have this power (Clarke and Becker, 2006). Studies have shown that extensive variability in these populations can help predict progression to malignancy (Merlo et al., 2006). Also, careful attention must be paid to the reliance on intensity. Referring to a high percentage of tetraploid cells as representative of an advanced form of disease may be misleading. Studies in our laboratory have shown the expression of p53/p73 and

mortalin in clams with <20% 4N cells but not in clams with higher ploidy levels in our lab (Siah, personal communication). According to other studies on hemic neoplasia in soft-shell clams, the expression of these proteins occurs in clams with high ploidy levels; intermediate stages are unknown in these studies (Kelley et al., 2001; Walker et al., 2006). Further studies at the earlier level of disease expression are anticipated to offer insight into hemic neoplasia in the soft- shell clam as each geographic situation and clam population may be unique.

This study has also contributed to the knowledge of the types of hemocytes in the soft-shell clam. This information is important due to the lack of studies on clam hemocytes and the lack of consensus among molluscan pathologists on both normal and neoplastic cell morphology. The descriptive information provided in this study may offer baseline information for recognizing cellular disorders such as benign and malignant neoplasia. In mammals, these are morphologically recognized by the cellular level of differentiation. Differentiation refers to the extent that neoplastic cells resemble normal cells on a functional and morphological level (Kumar et al., 2005). In mammalian medicine, the closer a neoplastic cell resembles its normal counterpart the more differentiated it is. A neoplastic cell that looks primitive and unspecialized is referred to as undifferentiated (Kumar et al., 2005). Malignant tumors are usually made up of the latter cells. Due to the lack of information on normal hemocytes in bivalves, determining the level of differentiation of a tumor population can be quite challenging.

In the soft- shell clam, neoplastic cells range in their level of possible differentiation with some appearing un-differentiated and primitive looking (Type B) while others appear moderately differentiated based on nuclear shape and size (Type A). In contrast to cells undergoing hyperplasia, dysplasia and metaplasia, neoplastic cells often more convincingly meet several of the criteria for anaplasia. Features of anaplasia include pleomorphism, abnormal nuclear morphology (increased DNA, hyperchromasia, increase in N:C ratio, presence of single or multiple nucleoli), bi-nucleate cells, mitotic figures and loss of polarity (Kumar et al., 2005). In the present study, neoplastic cells are found in high numbers infiltrating the entire clam body including the foot. This was interesting, as the foot mainly consists of muscle fibers and hemocytes are not typically located here. Bivalves respond to insults from irritants with an increase of hemocytes to the area of irritation or throughout the connective tissue of the body. However, neoplasia would be typically expected to cause a much more pronounced cellular infiltration. Neoplastic cells are seen in the connective tissues but also in the sinuses and muscle fibers, which is abnormal. Pathological descriptions found in the literature of hemic neoplasia include gill filament and connective tissue disruption (Cooper et al., 1982a). In the present study, enlarged gill filaments and disordered connective tissue were observed to a small degree. A small sample of clams was analyzed with histology in this study. A thorough examination of the process of cellular infiltration in tissues and organs in a larger number of clams is necessary to offer a thorough disease description of hemic neoplasia.

The lack of consensus among molluscan pathologists on criteria for neoplasia diagnosis has resulted in differences in interpretations and variable terminology used to describe neoplasia in bivalves. Investigators researching mollusc diseases as a group need to contribute to the development of a set of criteria for recognizing neoplasia in bivalves from other cellular disorders. In order to accomplish this goal, the use of mammalian terminology must be avoided. The bivalve system is much different from the mammalian system. Reliance on mammalian terminology not only prevents the development of terminology specific for bivalves but limits the understanding of bivalve physiology and pathology. Descriptions of neoplasia in bivalves began in the late 60's (Farley, 1969 a and b) and over 150 papers have been published (Barber, 2004). However, questions that were asked 30 years ago continue to be asked today. These include: What is/are the causes of neoplasia? Where do neoplastic cells originate and what are the criteria for recognizing them? What are the specific genes and proteins involved in neoplasia and their pathways? Is there a genetic susceptability to neoplasia? What role does prevalence and intensity have on mortality? While progress has been made for some of these questions, many remain unanswered.

Although the information provided in this study contributes to this knowledge, additional studies are essential to establish whether this is a true situation of neoplasia. Focus on the expression of specific genes or proteins that are more specific to neoplasia such as telomerase enzyme are needed. Improvement in immunophenotyping assays with the use of antibodies specific to neoplasia may help increase diagnostic sensitivity. In addition, the investigation of the functions of hemocytes in response to

environmental or pathogenic stress may offer insight into the regulation of certain genes and proteins in healthy and stressed clams that could further support the proliferative condition being studied as non-neoplastic or neoplastic. Studies on hemic neoplasia should take place on a yearly basis involving multiple sampling. This is important as a seasonal trend has been associated with hemic neoplasia and therefore disease expression may vary.

Large scale field and laboratory transmission studies would also offer insight into whether this disease can be transferred from clam to clam within a population. It is important that the use of true negative and positive clams are utilized in transmission studies, unlike past studies that have used "negative" clams from sites that have high prevalence and intensity. Further studies are required to determine the role that prevalence, intensity and their interaction contributes to population disease classification. The use of larger sampling sizes, more geographical sites and habitat comparisons may offer insight into the impact hemic neoplasia has on Atlantic Canada soft-shell clam populations.

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## Appendix A

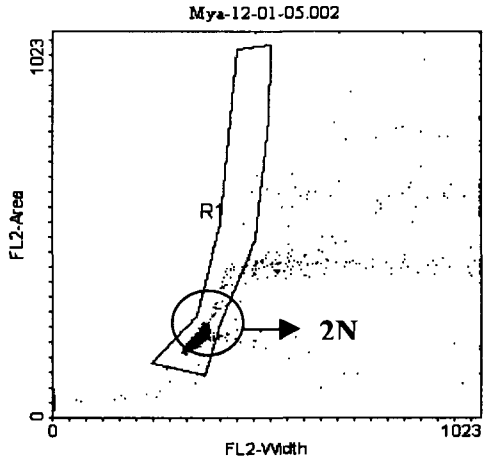



Figure 2a.

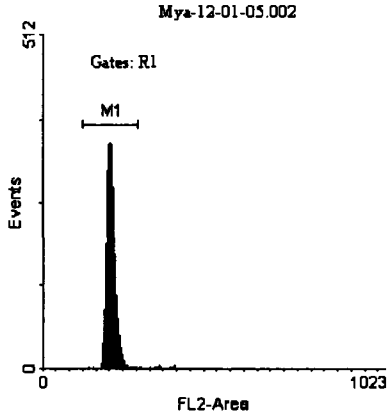



Figure 2b.

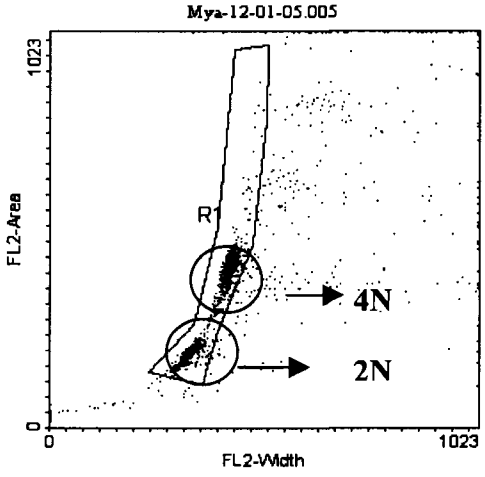



Figure 2c.

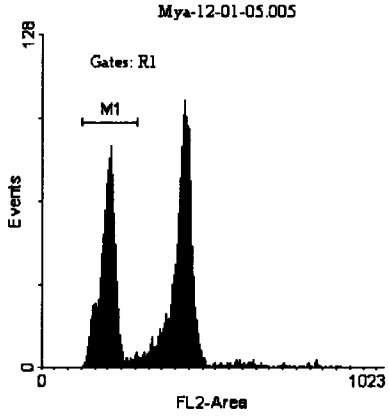



Figure 2d.

**Figure 2:** Graphical representation of hemic neoplasia negative and positive clams with flow cytometry. **(a)** Flow cytometry cytogram of a hemic neoplasia negative clam displaying single plot of diploid cells **(b)** Flow cytometry histogram of a hemic neoplasia negative clam displaying one major diploid peak **(c)** Flow cytometry cytogram of a hemic neoplasia positive clam displaying plots of both diploid cells and tetraploid cells **(d)** Flow cytometry histogram of hemic neoplasia positive clam displaying two peaks of diploid cells and tetraploid cells.

## Appendix B

**Table 2:** Field survey data (n=248 clams).

| Clam # | Site | FC 4N % | Cytology % of neoplastic cells |
|---|---|---|---|
| *2* | Prince Edward Island | 0.69 | 0 |
| *5* | Prince Edward Island | 0.75 | 0 |
| *6* | Prince Edward Island | 0.94 | 0.11 |
| *7* | Prince Edward Island | 0.94 | 0 |
| *8* | Prince Edward Island | 1.02 | 0 |
| *10* | Prince Edward Island | 1.07 | 0.49 |
| *22* | Prince Edward Island | 1.07 | 0 |
| *27* | Prince Edward Island | 1.12 | 0 |
| *31* | Prince Edward Island | 1.33 | 0 |
| *33* | Prince Edward Island | 1.5 | 0 |
| *37* | Prince Edward Island | 1.7 | 0.7 |
| *39* | Prince Edward Island | 1.74 | 0.32 |
| *41* | Prince Edward Island | 1.78 | 0.57 |
| *42* | Prince Edward Island | 1.82 | 0.12 |
| *43* | Prince Edward Island | 2.01 | 0.27 |
| *44* | Prince Edward Island | 2.04 | 0.23 |
| *49* | Prince Edward Island | 2.15 | 0.13 |
| *51* | Prince Edward Island | 2.2 | 0 |
| *59* | Prince Edward Island | 2.21 | 0.1 |
| *60* | Prince Edward Island | 2.22 | 0.21 |
| *309* | Prince Edward Island | 2.25 | 0.19 |
| *310* | Prince Edward Island | 2.26 | 0.2 |
| *311* | Prince Edward Island | 2.3 | 0.56 |
| *312* | Prince Edward Island | 2.34 | 0.56 |
| *313* | Prince Edward Island | 3.07 | 0.21 |
| *314* | Prince Edward Island | 3.14 | 0 |
| *315* | Prince Edward Island | 3.4 | 0.55 |
| *316* | Prince Edward Island | 3.4 | 0.51 |
| *317* | Prince Edward Island | 3.62 | 0.62 |
| *318* | Prince Edward Island | 3.9 | 1.2 |
| *319* | Prince Edward Island | 3.9 | 0.44 |
| *320* | Prince Edward Island | 3.93 | 0.31 |
| *321* | Prince Edward Island | 3.98 | 1.58 |
| *322* | Prince Edward Island | 4.07 | 0.62 |
| *323* | Prince Edward Island | 4.15 | 0.96 |
| *324* | Prince Edward Island | 4.4 | 1.64 |
| *325* | Prince Edward Island | 4.75 | 0.63 |
| *326* | Prince Edward Island | 5.85 | 1.8 |
| *327* | Prince Edward Island | 6.6 | 10.2 |
| *328* | Prince Edward Island | 6.61 | 8.5 |
| *329* | Prince Edward Island | 7.5 | 5.6 |
| *330* | Prince Edward Island | 8.32 | 12.1 |

| | | | |
|---|---|---|---|
| *331* | Prince Edward Island | 9.72 | 15.5 |
| *332* | Prince Edward Island | 10.09 | 5.63 |
| *333* | Prince Edward Island | 11.6 | 9.2 |
| *334* | Prince Edward Island | 12 | 10.1 |
| *335* | Prince Edward Island | 15.8 | 8.1 |
| *336* | Prince Edward Island | 20.1 | 37 |
| *337* | Prince Edward Island | 26.7 | 16.7 |
| *338* | Prince Edward Island | 28.2 | 33 |
| *342* | Prince Edward Island | 38 | 45 |
| *200* | Prince Edward Island | 44.6 | 30 |
| *169* | Prince Edward Island | 59 | 76 |
| *201* | Prince Edward Island | 68.5 | 42 |
| *343* | Prince Edward Island | 74.7 | 60 |
| *339* | Prince Edward Island | 82.7 | 72.8 |
| *202* | Prince Edward Island | 84.6 | 78.3 |

| **Clam #** | **Site** | **FC 4N %** | **Cytology % of neoplastic cells** |
|---|---|---|---|
| *1* | New Brunswick | 0.93 | 0 |
| *2* | New Brunswick | 1.31 | 0.1 |
| *3* | New Brunswick | 1.76 | 0.12 |
| *6* | New Brunswick | 1.76 | 0.12 |
| *7* | New Brunswick | 1.94 | 0.12 |
| *10* | New Brunswick | 1.96 | 0.1 |
| *11* | New Brunswick | 1.99 | 0.21 |
| *14* | New Brunswick | 2.03 | 0.31 |
| *15* | New Brunswick | 2.05 | 0.34 |
| *16* | New Brunswick | 2.62 | 1.2 |
| *19* | New Brunswick | 2.68 | 0.36 |
| *25* | New Brunswick | 2.74 | 0.1 |
| *26* | New Brunswick | 2.8 | 3.72 |
| *28* | New Brunswick | 3.41 | 0.04 |
| *29* | New Brunswick | 3.69 | 0.12 |
| *30* | New Brunswick | 3.76 | 0.43 |
| *32* | New Brunswick | 4.06 | 0.65 |
| *33* | New Brunswick | 4.31 | 0.62 |
| *36* | New Brunswick | 4.34 | 0.31 |
| *37* | New Brunswick | 4.62 | 0.65 |
| *38* | New Brunswick | 4.67 | 0.71 |
| *39* | New Brunswick | 5.41 | 1.11 |
| *40* | New Brunswick | 5.45 | 1.5 |
| *43* | New Brunswick | 5.66 | 1.21 |
| *48* | New Brunswick | 5.69 | 1.2 |
| *50* | New Brunswick | 5.94 | 3.23 |
| *51* | New Brunswick | 6.22 | 1.41 |
| *52* | New Brunswick | 6.25 | 3.78 |
| *55* | New Brunswick | 6.45 | 2.37 |

| | | | |
|---|---|---|---|
| *57* | New Brunswick | 7.1 | 5.97 |
| *58* | New Brunswick | 8.01 | 4.12 |
| ***Clam #*** | **Site** | **FC 4N %** | **Cytology % of neoplastic cells** |
| *2* | Magdalen Island | 0.42 | 0 |
| *4* | Magdalen Island | 0.48 | 0 |
| *5* | Magdalen Island | 0.55 | 0 |
| *6* | Magdalen Island | 0.76 | 0 |
| *8* | Magdalen Island | 1.01 | 0 |
| *11* | Magdalen Island | 1.01 | 0.15 |
| *14* | Magdalen Island | 1.12 | 0 |
| *15* | Magdalen Island | 1.21 | 0 |
| *19* | Magdalen Island | 1.22 | 0 |
| *21* | Magdalen Island | 1.54 | 0.47 |
| *22* | Magdalen Island | 1.59 | 0.2 |
| *24* | Magdalen Island | 1.64 | 0 |
| *25* | Magdalen Island | 1.66 | 0.1 |
| *27* | Magdalen Island | 1.78 | 0.16 |
| *29* | Magdalen Island | 1.8 | 0 |
| *30* | Magdalen Island | 1.89 | 0.32 |
| *31* | Magdalen Island | 1.99 | 0.02 |
| *32* | Magdalen Island | 2.16 | 0.2 |
| *35* | Magdalen Island | 2.29 | 0 |
| *36* | Magdalen Island | 2.29 | 0.41 |
| *37* | Magdalen Island | 2.63 | 0.42 |
| *38* | Magdalen Island | 2.66 | 0 |
| *39* | Magdalen Island | 3.04 | 0.28 |
| *40* | Magdalen Island | 3.23 | 0.12 |
| *41* | Magdalen Island | 3.29 | 1.25 |
| *42* | Magdalen Island | 3.65 | 0.3 |
| *43* | Magdalen Island | 3.7 | 1.12 |
| *44* | Magdalen Island | 4.28 | 0.81 |
| *45* | Magdalen Island | 4.33 | 0.65 |
| *46* | Magdalen Island | 6.02 | 1.31 |
| *51* | Magdalen Island | 6.66 | 2 |
| *52* | Magdalen Island | 7.2 | 3.81 |
| *53* | Magdalen Island | 7.45 | 3.67 |
| *54* | Magdalen Island | 8.01 | 15.4 |
| *55* | Magdalen Island | 9.15 | 12.5 |
| *58* | Magdalen Island | 13.5 | 7.1 |
| *60* | Magdalen Island | 14.4 | 19.2 |

| Clam # | Site | FC 4N % | Cytology % of neoplastic cells |
|---|---|---|---|
| *1* | Newfoundland | 0.91 | 0 |
| *2* | Newfoundland | 1.11 | 0 |
| *5* | Newfoundland | 1.26 | 0 |
| *7* | Newfoundland | 1.33 | 0 |
| *8* | Newfoundland | 1.48 | 0 |
| *11* | Newfoundland | 1.7 | 0.23 |
| *13* | Newfoundland | 1.75 | 0.1 |
| *16* | Newfoundland | 1.78 | 0.21 |
| *17* | Newfoundland | 2.01 | 1.1 |
| *18* | Newfoundland | 2.1 | 0.56 |
| *19* | Newfoundland | 2.12 | 0.78 |
| *20* | Newfoundland | 2.23 | 0.12 |
| *21* | Newfoundland | 2.28 | 0.23 |
| *23* | Newfoundland | 2.43 | 1.21 |
| *24* | Newfoundland | 2.45 | 0.35 |
| *25* | Newfoundland | 2.71 | 0.4 |
| *27* | Newfoundland | 2.85 | 0.25 |
| *28* | Newfoundland | 2.9 | 0.31 |
| *29* | Newfoundland | 3.12 | 1.39 |
| *31* | Newfoundland | 3.19 | 0.27 |
| *32* | Newfoundland | 3.84 | 0.37 |
| *33* | Newfoundland | 3.87 | 0.81 |
| *35* | Newfoundland | 4.87 | 0.67 |
| *36* | Newfoundland | 5.24 | 1.34 |
| *37* | Newfoundland | 5.3 | 1.75 |
| *38* | Newfoundland | 5.33 | 1.32 |
| *40* | Newfoundland | 5.43 | 2.87 |
| *42* | Newfoundland | 5.66 | 1.5 |
| *44* | Newfoundland | 6.17 | 2.24 |
| *45* | Newfoundland | 6.33 | 2.65 |
| *46* | Newfoundland | 6.68 | 3.12 |
| *47* | Newfoundland | 7.01 | 2.28 |
| *49* | Newfoundland | 7.67 | 2.84 |
| *50* | Newfoundland | 8.25 | 4.9 |
| *51* | Newfoundland | 8.45 | 5.5 |
| *52* | Newfoundland | 9.29 | 7.4 |
| *53* | Newfoundland | 9.38 | 2.41 |
| *54* | Newfoundland | 9.5 | 3.81 |
| *55* | Newfoundland | 9.59 | 3.89 |
| *56* | Newfoundland | 10.1 | 3.58 |
| *57* | Newfoundland | 13.19 | 8.6 |
| *59* | Newfoundland | 16.4 | 9.31 |

| Clam # | Site | FC 4N % | Cytology % of neoplastic cells |
|---|---|---|---|
| *1* | Saguenay Bay, QC | 0.08 | 0 |
| *6* | Saguenay Bay, QC | 0.39 | 0 |
| *7* | Saguenay Bay, QC | 0.43 | 0 |
| *8* | Saguenay Bay, QC | 0.5 | 0 |
| *9* | Saguenay Bay, QC | 0.56 | 0 |
| *10* | Saguenay Bay, QC | 0.58 | 0 |
| *11* | Saguenay Bay, QC | 0.6 | 0 |
| *14* | Saguenay Bay, QC | 0.69 | 0 |
| *15* | Saguenay Bay, QC | 0.84 | 0 |
| *16* | Saguenay Bay, QC | 0.89 | 0 |
| *17* | Saguenay Bay, QC | 0.9 | 0 |
| *18* | Saguenay Bay, QC | 1.01 | 0 |
| *19* | Saguenay Bay, QC | 1.07 | 0.9 |
| *20* | Saguenay Bay, QC | 1.13 | 0.19 |
| *25* | Saguenay Bay, QC | 1.13 | 0.1 |
| *26* | Saguenay Bay, QC | 1.18 | 0.26 |
| *28* | Saguenay Bay, QC | 1.2 | 0 |
| *30* | Saguenay Bay, QC | 1.21 | 0 |
| *32* | Saguenay Bay, QC | 1.21 | 0.27 |
| *33* | Saguenay Bay, QC | 1.26 | 0.23 |
| *37* | Saguenay Bay, QC | 1.4 | 0.34 |
| *38* | Saguenay Bay, QC | 1.5 | 0.1 |
| *39* | Saguenay Bay, QC | 1.5 | 0.12 |
| *40* | Saguenay Bay, QC | 1.62 | 0.26 |
| *42* | Saguenay Bay, QC | 1.72 | 0 |
| *43* | Saguenay Bay, QC | 1.73 | 0.1 |
| *44* | Saguenay Bay, QC | 1.75 | 0.24 |
| *45* | Saguenay Bay, QC | 1.87 | 0.41 |
| *46* | Saguenay Bay, QC | 1.96 | 0.46 |
| *51* | Saguenay Bay, QC | 2.01 | 0.5 |
| *53* | Saguenay Bay, QC | 2.4 | 0.29 |
| *54* | Saguenay Bay, QC | 2.49 | 0.32 |
| *55* | Saguenay Bay, QC | 2.55 | 0.35 |
| *57* | Saguenay Bay, QC | 2.58 | 0.32 |
| *58* | Saguenay Bay, QC | 3.61 | 0.65 |
| *60* | Saguenay Bay, QC | 4.15 | 0.72 |
| **Clam #** | **Site** | **FC 4N %** | **Cytology % of neoplastic cells** |
| *2* | Metis Bay, QC | 0.23 | 0 |
| *3* | Metis Bay, QC | 0.47 | 0 |
| *4* | Metis Bay, QC | 0.52 | 0 |
| *5* | Metis Bay, QC | 0.55 | 0 |
| *6* | Metis Bay, QC | 0.59 | 0 |
| *7* | Metis Bay, QC | 0.79 | 0 |
| *8* | Metis Bay, QC | 0.79 | 0 |
| *9* | Metis Bay, QC | 0.96 | 0 |

| | | | |
|---|---|---|---|
| *10* | Metis Bay, QC | 0.99 | 0 |
| *12* | Metis Bay, QC | 1.08 | 0.12 |
| *13* | Metis Bay, QC | 1.08 | 0 |
| *14* | Metis Bay, QC | 1.27 | 0.12 |
| *15* | Metis Bay, QC | 1.27 | 0.48 |
| *16* | Metis Bay, QC | 1.27 | 0.27 |
| *17* | Metis Bay, QC | 1.3 | 0 |
| *18* | Metis Bay, QC | 1.3 | 0 |
| *19* | Metis Bay, QC | 1.33 | 0.23 |
| *20* | Metis Bay, QC | 1.41 | 0 |
| *21* | Metis Bay, QC | 1.9 | 0.88 |
| *23* | Metis Bay, QC | 1.9 | 0 |
| *24* | Metis Bay, QC | 2.34 | 0.23 |
| *26* | Metis Bay, QC | 2.63 | 1.2 |
| *27* | Metis Bay, QC | 2.68 | 0.45 |
| *28* | Metis Bay, QC | 2.81 | 1.23 |
| *30* | Metis Bay, QC | 2.91 | 0.34 |
| *31* | Metis Bay, QC | 2.91 | 0 |
| *32* | Metis Bay, QC | 2.93 | 0.1 |
| *34* | Metis Bay, QC | 2.93 | 0.31 |
| *36* | Metis Bay, QC | 3.03 | 0.76 |
| *38* | Metis Bay, QC | 3.23 | 0.1 |
| *39* | Metis Bay, QC | 3.23 | 0.17 |
| *40* | Metis Bay, QC | 3.8 | 0.38 |
| *42* | Metis Bay, QC | 3.95 | 0.4 |
| *45* | Metis Bay, QC | 4.02 | 0.51 |
| *46* | Metis Bay, QC | 4.29 | 1.26 |
| *47* | Metis Bay, QC | 4.7 | 0.49 |
| *48* | Metis Bay, QC | 4.78 | 0.76 |
| *54* | Metis Bay, QC | 5.28 | 2.5 |
| *55* | Metis Bay, QC | 5.38 | 1.62 |
| *56* | Metis Bay, QC | 6.45 | 2.8 |
| *57* | Metis Bay, QC | 6.45 | 3.48 |
| *58* | Metis Bay, QC | 7.13 | 2.9 |
| *59* | Metis Bay, QC | 10 | 16.2 |
| *60* | Metis Bay, QC | 15.5 | 23 |

**Appendix C1**

**Table 2**: Summary of differential hemocyte count (±SD) for Havre-aux-Maisons, Magdalen Islands, QC, (n= 40 clams (4000 cells)).

| **Cell Types** | **Differential count %** | **Differential range %** |
|---|---|---|
| Type 1 Granulocyte | 29 (± 9.1) | 14 - 51 |
| Type 2 Granulocyte | 6 (± 4.9) | 0 - 26 |
| Type 1 Agranulocyte | 43.7 (± 13.2) | 20 - 67 |
| Type 2 Agranulocyte | 15.9 (± 13.1) | 0 - 53 |
| Type 3 Agranulocyte | 2.8 (± 3.6) | 0 - 15 |
| Disintegrated Cells | 2.6 (± 2.3) | 0 - 8 |

Mean total hemocyte count - 4.7 (± 0.48) x $10^6$/ ml (range- 3.3 - 5.5 x $10^6$ / ml)

**Table 3**: Summary of hemocyte absolute count (±SD) for Havre-aux-Maisons, Magdalen Islands, QC (n = 40 clams (4000 cells)).

| **Cell Types** | **Absolute count** (x $10^5$/ml) | **Absolute range** (x $10^5$/ml) |
|---|---|---|
| Type 1 Granulocyte | 13 (± 4.4) | 6.6 - 27 |
| Type 2 Granulocyte | 3 (± 2.3) | 0 - 10 |
| Type 1 Agranulocyte | 21 (± 6.8) | 9.4 - 32 |
| Type 2 Agranulocyte | 7.5 (± 6.3) | 0 - 25 x |
| Type 3 Agranulocyte | 1.3 (± 1.8) | 0 - 7.5 |
| Disintegrated Cells | 1.2 (± 1.1) | 0 - 4.1 |

**Appendix C2**

**Table 4**: Summary of differential hemocyte count (±SD) for North River, PEI, (n= 40 clams (4000 cells).

| Cell Types | Differential count % | Differential range % |
|---|---|---|
| Type 1 Granulocyte | 21.8 (± 10.5) | 0 - 40 |
| Type 2 Granulocyte | 2.9 (± 3.9) | 0 -15 |
| Type 1 Agranulocyte | 32 (± 24.4) | 0 - 83 |
| Type 2 Agranulocyte | 10.2 (± 14.1) | 0 - 62 |
| Type 3 Agranulocyte | 0.95 (± 2.3) | 0 -10 |
| Neoplastic (A and B) | 28.9 (± 26.7) | 0 - 91 |
| Disintegrated Cells | 3.3 (± 2.9) | 0 - 8 |

Mean total hemocyte count - 4.6 (± 0.91) x $10^6$/ml (range - 3.5 - 7.5 x $10^6$/ml)

**Table 5**: Summary of hemocyte absolute count (±SD) for North River, PEI, (n = 40 clams (4000 cells).

| Cell Types | Absolute count (x $10^5$ /ml) | Absolute range (x $10^5$/ml) |
|---|---|---|
| Type 1 Granulocyte | 10 (± 5.3) | 0 - 22 |
| Type 2 Granulocyte | 1.2 (± 1.7) | 0 - 6.5 |
| Type 1 Agranulocyte | 14 (± 9.9) | 0 - 37 |
| Type 2 Agranulocyte | 4.3 (± 5.9) | 0 - 27 |
| Type 3 Agranulocyte | 0.39 (± 0.92) | 0 - 4.1 |
| Neoplastic (A and B) | 15 (± 16) | 0 - 66 |
| Disintegrated Cells | 1.4 (± 1.3 | 0 - 3.8 |

**Table 6**: Summary of North River, PEI - Differntial Hemocyte Count (DHC) (±SD) and absolute hemocyte count (±SD) in 12 positive clams (50 - 84 % 4N).

| Cell Type | DHC % | Range | Absolute count ( x $10^5$/ ml) | Range ( x $10^5$/ ml) |
|---|---|---|---|---|
| Type 1 Granulocyte | 25.3 (± 8.7) | 5 - 35 | 14 (± 4.9) | 3.7 – 22 |
| Type 2 Granulocyte | 2.4 (± 3.3) | 0 - 10 | 1.3 (± 1.9) | 0.0 - 5.5 |
| Type 1 Agranulocyte | 10.8 (± 10.7) | 0 - 29 | 5.6 (± 5.5) | 0.0 – 16 |
| Type 2 Agranulocyte | 2.9 (± 5.6) | 0 -18 | 1.6 (± 0.28) | 0.0 - 9.0 |
| Type 3 Agranulocyte | 0 | 0 | 0 | 0 |
| Neoplastic (A and B) | 56.7 (± 17.1) | 38 - 91 | 33 (±14.7) | 14 – 66 |
| Distingrated | 1.7 (± 2.3) | 0 - 7 | 0.94 (± 1.2) | 0.0 - 3.8 |

Mean total hemocyte count: 5.6 (± 1.1) x $10^6$/ ml (range: 3.8 - 7.5 x $10^6$/ ml)

## Appendix D1

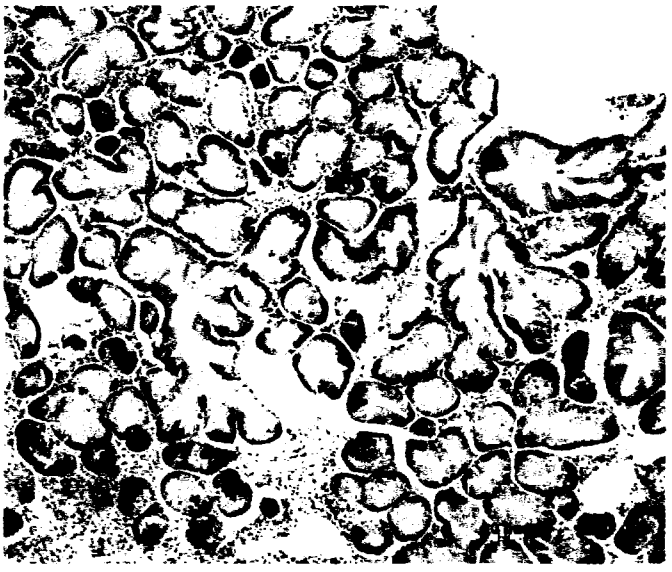
**Table 7:** Histological analysis of soft-shell clams from North River, PEI.

| North River, PE Clam # | FC 4N % | Mantle | Gills | Gonad | Digestive Gland | Kidney | Sinuses | Foot | Mitotic Figs |
|---|---|---|---|---|---|---|---|---|---|
| ***Group 1 (<50% 4N)*** | | | | | | | | | |
| 550 | **8** | _ | _ | _ | _ | _ | _ | _ | _ |
| 521 | **10** | _ | _ | _ | _ | _ | _ | _ | _ |
| 518 | **12** | _ | _ | _ | Light | _ | _ | _ | _ |
| 487 | **14** | _ | Light | _ | _ | _ | _ | _ | _ |
| 481 | **18** | _ | Light | _ | Light | _ | _ | _ | _ |
| 561 | **21** | _ | Light | _ | Light | _ | _ | _ | _ |
| 563 | **24** | _ | Light | Light | Light | Light | _ | _ | _ |
| 249 | **35** | Moderate | Moderate | _ | Moderate | _ | _ | _ | _ |
| 917 | **42** | _ | Moderate | Light | Light | _ | _ | _ | 1-2 gills |
| 330 | **45** | Moderate | Moderate | Light | Moderate | Light | Present | _ | _ |
| ***Group 2 (>50% 4N)*** | | | | | | | | | |
| 309 | **53** | Moderate | Light | Moderate | Moderate | _ | Present | _ | 1-2 foot |
| 551 | **56** | Moderate | Moderate | Moderate | Moderate | Light | _ | Light | _ |
| 333 | **59** | _ | Moderate | Heavy | Moderate | Light | _ | _ | _ |
| 851 | **63** | Moderate | Moderate | Moderate | Moderate | Light | _ | Moderate | _ |
| 853 | **68** | Moderate | Heavy | Moderate | Heavy | _ | _ | Heavy | 1-2 mantle |
| 846 | **75** | Heavy | Heavy | Moderate | Heavy | Light | _ | Moderate | _ |
| 776 | **79** | Moderate | Heavy | Moderate | Heavy | Light | _ | Heavy | _ |
| 727 | **82** | Heavy | Heavy | Heavy | Heavy | Light | Present | Heavy | _ |
| 946 | **84** | Heavy | Heavy | Heavy | Heavy | Light | Present | Heavy | 3-5 foot |
| 882 | **88** | Heavy | Heavy | Heavy | Heavy | Light | Present | Heavy | _ |

Histology scale (estimation of percentage of neoplastic cells of total tissue cells): Light- < 20% neoplastic cells, Moderate - >20% <50% neoplastic cells, Heavy - 50% + neoplastic cells

**Appendix D2**

a.



b.

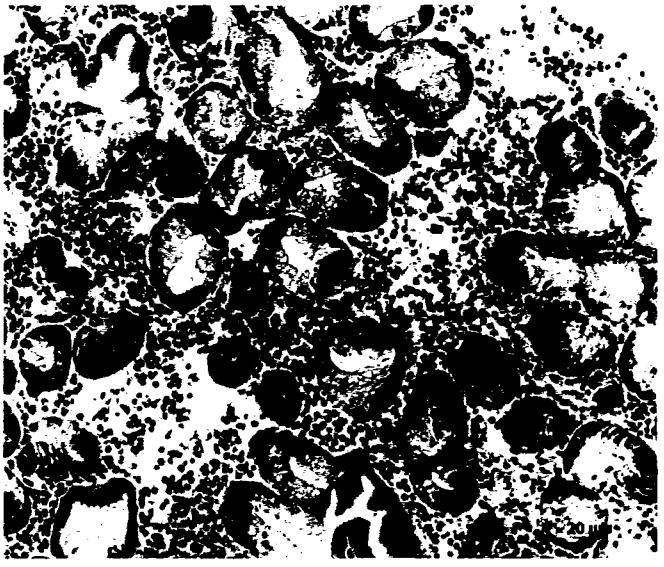


**Figure 5:** Histological section of soft - shell clams, *Mya arenaria* (a) Hemic neoplasia negative clam. Section of digestive gland with normal branched ducts and tubules (b) Hemic neoplasia positive clam. Section of digestive gland with heavy infiltration of neoplastic cells. (40x) scale bar = 20 μm